TOBACCO RELATED DISEASE RESEARCH PROGRAM
GRANT APPLICATION
Follow instructions carefully.

2 IT - 0 0 0 71

1. TITLE OF PROJECT (Do not exceed 60 typewriter spaces.)
Human Markers for (S)-Nicotine Biotransformation and Pathology

2. TYPE OF APPLICATION: [ ] RESEARCH [X] IDEAS

3. PREFERRED STUDY SECTION (See page 5)
General Biomedical

4a. PRINCIPAL INVESTIGATOR (Last name, first name, middle name)
Cashman, John Richard

4b. POSITION TITLE
Assistant Professor

4c. DEGREES
Ph.D.

4d. DEPARTMENT, SERVICE, LABORATORY, or EQUIVALENT
Pharmaceutical Chemistry

4e. MAILING ADDRESS (Street, city, state, zip code)
University of California, San Francisco
School of Pharmacy
Department of Pharmaceutical Chemistry
Box 0446
513 Parnassus Avenue
San Francisco, CA 94143

RECEIVED
JAN 15 1991

4f. MAJOR SUBDIVISION
School of Pharmacy

4g. CONTACT TELEPHONE (Area code, number, and extension)
415-476-1916

4h. FAX NO.
415-476-0688

5a. VERTEBRATE ANIMALS: [X] NO [ ] YES — IACUC approved date — Assurance No.

5b. HUMAN SUBJECTS: [ ] NO [X] YES — pending — M 1169

5c. BIOHAZARDS: [ ] NO [X] YES — 03/28/90

6. DURATION OF PROPOSED PROJECT: [X] 1 [ ] 2 [ ] 3 YEARS

7a. DIRECT COSTS REQUESTED FOR ENTIRE PROJECT PERIOD: $75,000

7b. TOTAL COST REQUESTED FOR ENTIRE PROPOSED PROJECT PERIOD: $75,000

8. NAME OF APPLICANT ORGANIZATION
Regents, University of California
ADDRESS (Street, city, state, and zip code)
Office of Research Affairs
University of California, San Francisco
3333 California Street, Suite 11
San Francisco, CA 94143-0962

9. PERFORMANCE SITES (Organization and address)
University of California, San Francisco
School of Pharmacy
San Francisco, CA 94143

10. TYPE OF ORGANIZATION: [X] Public [ ] Federal [X] State [ ] Local [ ] Private (Nonprofit)

11. COUNTY
San Francisco

12. NAME OF ADMINISTRATIVE CONTACT
Georgia Ann C. Hansen
TELEPHONE (Area code, number, and extension)
415-476-2611
TITLE
Contracts and Grants Coordinator
ADDRESS
University of California, San Francisco
School of Pharmacy, Dean's Office, Box 1204
San Francisco, CA 94143-1204

13. NAME OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Lorraine M. Petrakis
TELEPHONE (Area code, number, and extension)
415-476-2977
TITLE
Director, Office of Research Affairs
ADDRESS
University of California, San Francisco
3333 California Street, Suite 11
San Francisco, CA 94143-0962

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 4a. (In ink. "per" signature not acceptable)
John R Cashman
DATE Jan 7, 1991

15. CERTIFICATION AND ACCEPTANCE: I certify that the statement herein are true and complete to the best of my knowledge, and accept the obligation to comply with Tobacco-Related Disease Research Program terms and conditions if a grant is awarded as the result of this application.

SIGNATURE OF PERSON NAMED IN 13. (In ink. "per" signature not acceptable)
DATE JAN 14 1991

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Principal Investigator: ohn R. Cashman

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XIII. Appendix (no page numbering required)
- Copies of Assurances
- Indirect Cost Agreement (if applicable)
- Supporting materials

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Principal Investigator: John R. Cashman

ABSTRACT

Human Markers for (S)-Nicotine Biotransformation and Pathology

Title of Research Project (do not exceed 60 spaces)

Department of Pharmaceutical Chemistry, University of California, San Francisco

Department and Institution

A relation between smoking and human disease has been clearly established, although a role for nicotine in this process has yet to be fully defined. Nicotine causes complex central nervous system, behavioral, cardiovascular, endocrine, neuromuscular and metabolic effects in humans. It is clear that smoking is a major risk factor for coronary and peripheral vascular disease. Although nicotine itself is not carcinogenic, nicotine-derived nitrosamines and other metabolites may be the causative agents for cancer in humans exposed to nicotine. A complete characterization of the health risk of nicotine requires the quantification of the metabolism, disposition, pharmacokinetics and genetic predisposition of a smoker to toxicating and detoxicating routes of metabolism. The balance between detoxication and toxication nicotine metabolic pathways may predispose humans to the pathological consequences of smoking. It is the hypothesis of this proposal that the **differences in the rate of metabolism of nicotine underlie the susceptibility of humans** to the pathological effects of nicotine. A thorough molecular knowledge of the toxicating and detoxicating pathways of nicotine metabolism may provide a fundamental basis for determining the relative susceptibility of humans to the pathological effects of tobacco.

In collaboration with Professor Neal Benowitz and Dr. Peyton Jacob III of UCSF we shall specifically test this hypothesis. The studies are divided into two sections: **1) Determination of the stereochemistry of the major detoxication route of (S)-nicotine metabolism, namely (S)-nicotine N'-oxide, determination of the (S)-cotinine:(S)-nicotine N'-oxide ratio and the overall pharmacokinetic profile for deuterium labeled (S)-nicotine infused in healthy smokers, and 2) Determination of the molecular basis for (S)-nicotine N'-oxide formation in humans by analysis of detoxication enzymes by Northern and Southern blot analysis of blood of healthy smokers infused with deuterium labeled (S)-nicotine.**

We shall stereoselectively synthesize *cis*- and *trans* (S)-nicotine N'-oxides by a novel procedure recently developed in our laboratory. We shall use these standards to analyze the blood and urine of healthy smokers infused with deuterium labeled (S)-nicotine for the stereochemistry of (S)-nicotine N'-oxide formation and thus deduce the involvement of the flavin-containing monooxygenase (FMO) in (S)-nicotine detoxication. The pharmacokinetics of (S)-nicotine, (S)-cotinine and (S)-nicotine N'-oxide disposition will be determined by novel, recently developed gas chromatography (GC) and GC-mass spectrometry analytical techniques. We shall obtain cDNA probes recently made available in our laboratory to perform Northern and Southern blot analysis of blood of smokers to quantify the mRNA levels and gene copies of human lung and liver FMO. Knowledge of the (S)-cotinine to (S)-nicotine N'-oxide ratio coupled with FMO levels may lead to general strategies for predicting human susceptibility to the pathological consequences of nicotine.

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RELEVANCE STATEMENT

Describe briefly the importance of your research to tobacco-related disease and what impact your research might have on these diseases and the California population at risk. Do not exceed one page.

Approximately 30% of adult Californians smoke despite convincing knowledge of the health hazards of smoking (1). A relation between smoking and human disease (central nervous system, behavioral, cardiovascular, endocrine, neuromuscular, metabolic, coronary and peripheral vascular diseases as well as cancer) has been clearly established, although a role for nicotine in this process has yet to be fully defined (2).

Careful pharmacokinetic investigations of humans infused with stable isotope-labeled nicotine by Professor N.L. Benowitz and Dr. Peyton Jacob III (10-12) combined with highly sensitive analytical methods developed in their laboratory (22) have shown that for Californians, only 3-5% of (S)-nicotine is identifiable as (S)-nicotine N'-oxide. In contrast, research groups working in the United Kingdom have reported as much as 60% of a dose of (S)-nicotine is converted to (S)-nicotine N'-oxide (19). This observation, coupled with the fact that only 35-40% of ingested nicotine is accounted for (in Californians) in terms of identifiable metabolites, presents a most challenging task for risk analysis of the effect of nicotine metabolism on human disease. These facts point out the need for nicotine metabolism studies employing a reliable method to quantify administration and elimination of drug in humans.

The fact that Gorrod *et al.* determined that a high cotinine:nicotine N'-oxide ratio was positively correlated to bladder cancer in human cigarette smokers bears on the current controversy surrounding (S)-nicotine N'-oxide formation in Californians and the British. Because we and others (30,54) postulate that flavin-containing monooxygenase catalyzed (S)-nicotine N'-oxygenation is the major route of detoxication for nicotine, the large discrepancy between Californian and British nicotine N'-oxide levels may suggest that Californians with high cotinine:nicotine N'-oxide ratios are particularly susceptible to the pathological effects of cigarette smoking.

Finally, the fact that only 35-40% of ingested nicotine is accounted for in terms of identifiable metabolites points out the need for careful metabolic and elimination studies in humans.

Estimation of the intake and metabolism of nicotine by tobacco users is of fundamental use to researchers studying smoking behavior, as well as epidimiologists and clinicians assessing health risks. Because it is likely that human genetic polymorphism exists for FMO (54) and genetic deficiency of FMO may make humans especially susceptible to the pathological effects of nicotine (30) it is important to evaluate smokers for the presence of FMO. With the recent cloning and sequencing of animal and human liver and lung FMO we can now assess the contribution of FMO-mediated nicotine N'-oxygenation to the overall metabolism and pathology of nicotine.

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Principal Investigator: John R. Cashman

KEY PERSONNEL

Name, Degree(s)	Position Title and Project Role	Department and Organization
John Cashman, Ph.D.	Principal Investigator	Department of Pharmaceutical Chemistry, UCSF
Neal Benowitz, M.D.	Co-investigator	Division of Clinical Pharmacology, UCSF
Peyton Jacob III, Ph.D.	Co-investigator	Division of Clinical Pharmacology, UCSF
Sang Bum Park, Ph.D.	Postdoctoral Fellow	Department of Pharmaceutical Chemistry, UCSF
Joelle Thomas, M.S., DEA.	Postgraduate Researcher	Department of Pharmaceutical Chemistry, UCSF
Patricia Buley, B.S.	Research Assistant	Division of Clinical Pharmacology, UCSF
David Williams, Ph.D.	Consultant	Oregon State University
Ronald Hines, Ph.D.	Consultant	Wayne State University

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Principal Investigator: Cashman, John R.

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD DIRECT COSTS ONLY				
BUDGET CATEGORY TOTALS		YEAR 1	YEAR 2	YEAR 3
PERSONNEL (Salary and fringe benefits) (Applicant organization only)		REDACTED		
CONSULTANT and CONTRACTUAL COSTS		0		
EQUIPMENT		5,000		
SUPPLIES & EXPENSES		19,200		
DOMESTIC TRAVEL	PROJECTED RELATED			
	SCIENTIFIC MEETINGS	1,000		
TOTAL DIRECT COSTS		A 75,000	B	C
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD (Item 7) (D = Total of A, B, and C)				$ 75,000 D

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Principal Investigator: Cashman, John R.

DETAILED BUDGET FOR YEAR 01 BUDGET PERIOD DIRECT COSTS ONLY					FROM 07/01/91	THROUGH 06/30/92	
PERSONNEL (Applicant organization only)		1 % FTE Appt.	2 % FTE on Project	3 % FTE Salary Request	DOLLAR AMOUNT REQUESTED (Omit cents)		
NAME	Role in Project				SALARY	FRINGE BENEFITS	TOTALS
JR Cashman	Principal Investigator	1.00	5%	5%			
N Benowitz	Co-Investigator	1.00	2.50%	0%			
P Jacob III	Assoc. Res. Chemist	1.00	2.50%	0%			
SB Park	Postdoctoral Fellow	1.00	100%	100%			
J Thomas	Research Assistant	0.50	80%	40%			
P Buley	Research Assistant	0.50	10%	5%			
Fringe rates:	Academic	23.50%					
Fringe rate:	Research Assistants	4.66%					
SUBTOTALS →							

REDACTED

CONSULTANT AND CONTRACTUAL COSTS				
EQUIPMENT (Itemize)				
LKB Model 2297 Macrodrive 5 Power Supply	3,000			
Refrigerator (spark proof)	800			
Haake Water Bath Circulator	1,200			5,000
SUPPLIES AND EXPENSES (Itemize by category)				
Radiosotopes	1,000	Radiation Safety Supplies and Disposal	1,600	
Kits for Genetics	800			
Chemicals, Cofactors, Enzymes	600	Chemical Synthesis	1,000	
Nitrocellutose, Blots and Sheets	200	NMR Time and Supplies	300	
X-ray and Polaroid Film	200	Pipettmen	1,000	
Electrophoresis Box for Minigels	300	Oligonucleotide Synthesis	1,500	
Enzymes for Genetics	600	Mass Spectrometry Expenses	4,000	
Glassware and Plasticware	400	Maintenance and Repair	800	
HPLC Supplies, Columns	2,600	Duplication, Telephone	500	
Electrophoresis Supplies	1,000	Publication Costs	600	
		Office Supplies	200	19,200
TRAVEL	PROJECTED-RELATED			
	SCIENTIFIC MEETING(S)	PI & Postdoct to attend scientific meetings		1,000
TOTAL DIRECT COST FOR YEAR_01_BUDGET PERIOD →			$	75,000

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BUDGET JUSTIFICATION

(see Section VII of Instructions)

PERSONNEL

Principal Investigator. Study of the enzyme mechanism and molecular aspects of the human flavin-containing monooxygenase constitutes the major research project in our laboratory. Although the research focuses on a single important human metabolite of (S)-nicotine, the experimental approaches involved will range from genetics to chemical synthesis. The PI shall be heavily involved with this project and requests 5% of his salary. The consultants have not requested any additional support fees. The PI has long standing collaborations with the consultants and routinely shares reagents, assays and expertise. He will supervise the project and direct Dr. Park and Ms. Thomas.

Co-Investigator. Professor N.L. Benowitz is a nationally and internationally known clinician who has studied the pharmacological, metabolic, behavioral and chemical effects of nicotine on humans. He will be responsible for recruiting the subjects, administering the (S)-nicotine and obtaining blood and urine samples. Over 100 humans have been infused with stable isotope labeled (S)-nicotine in his laboratory. The human protocol outlined herein comes directly from a human protocol that is approved and operational in his laboratory. We have both had a long standing interest in the N-oxygenation of tertiary amines and we look forward to continuing our work in this area.

Co-Investigator. Dr. Peyton Jacob III is a nationally renowned medicinal chemist who has pioneered many chemical and metabolic studies of nicotine. His responsibilities will include the design and execution of strategies required for the GC and GCMS analysis of (S)-nicotine and nicotine metabolites. He will supervise the work of Patricia Buley in the acquisition and interpretation of routine pharmacokinetic analysis of infused d_2-(S)-nicotine. He will synthesize and oversee the sterilization and purity of d_2-(S)-nicotine.

Postdoctoral Fellow. Dr. Sang Bum Park is a medicinal chemist who has been a postdoc in my laboratory for the last 5 months. In that time he has very ably stereoselectively synthesized S-oxides and N-oxides of drugs and chemicals by the modified Sharpless method and by enzymatic procedures. He has done metabolism studies and developed new HPLC methods. His responsibilities will include: a) stereoselective synthesis and characterization of (S)-nicotine N'-oxides by NMR, CD, MS, UV-vis, IR and HPLC, b) metabolism of (S)-nicotine in human liver and lung microsomes and highly purified FMO and other enzyme preparations, and c) routine HPLC analysis of metabolic extracts from human blood and urine.

Postgraduate Researcher. Ms. Joelle Thomas is a visiting researcher from France. She has extensive experience in the protein chemistry and molecular biology of monooxygenases. Currently, she is performing molecular biology studies of FMO in my laboratory. Her responsibilities shall include: a) transfection, production and sequencing of human liver and lung FMO cDNA oligonucleotide probes for use in Northern and Southern blot analysis, b) Northern and Southern blot analysis of mRNA blood from cigarette smokers infused with nicotine, and c) other microsome or protein purification as needed for Dr. Sang Bum Park (described above).

Research Assistant. Ms. Patricia Buley is a research assistant in the laboratory of Drs. Benowitz and Jacob. She runs the GC and mass spectrometer for the routine pharmacokinetic analysis of extracted human blood and urine samples. Her responsibilities shall include a) extraction of blood and urine samples of humans dosed with d_2-(S)-nicotine and b) GC and GCMS analysis of extracts of human blood and urine for (S)-nicotine, (S)-cotinine, (S)-nicotine N'-oxide (and possibly 3-hydroxycotinine).

EQUIPMENT

A minimal amount of equipment is requested to augment the equipment currently on hand. A Haake water bath ($1,200) is required for use with our water-cooled Macrophor DNA electrophoresis unit required

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for mRNA. The pump that we currently use is old and unreliable. A new refrigerator/freezer dedicated to blood and urine samples is requested. Our current refrigerator/freezer unit is old and unreliable and we need to avoid contamination or degradation of these precious samples.

Expendable Supplies. A minimal amount of basic supplies are requested for use in our studies. Radioisotopes for nick translation, sequencing and Southern and Northern blots will be extensive: α [^{32}P] – dNTPs, α[^{35}S] sATP, α[^{32}P]-ATP and [^{35}S]-methionine will cost $1,000 per year. In connection with our use of radioisotopes are associated scintillation supplies including cocktail, vials and standards ($400), radiation safety protection gloves, lab coats, goggles, shields ($200) and radiation disposal (1,000) at $51/cu ft dry waste, ($15/tray) and liquid waste ($20/gal) are expected to increase 10%. We shall make extensive use of chemicals and enzymes for genetic work including: restriction endonucleases, T4-polynucleotide kinase, DNA polymerase, DNA ligase, terminal transferase, Klenow fragment, RNAse, reverse transcriptase, etc. for a total of $600 . A number of expensive genetic kits available for nick translation, random priming, sequencing, transcription, translation and other cloning kits will cost ($800). A number of expensive high quality chemicals including: IPTG, X-Gal, TRIS, agarose, glycerol will cost ($400) and cofactors NADPH, FAD, etc. and enzymes will cost ($200) per year. Because we will perform a large number of Northern and Southern blots, nitrocellulose blots and sheets will cost ($200). X-ray and polaroid film will be essential for DNA sequencing, Northern and Southern blots and cloning and subcloning and will cost ($200). Disposable plasticware and glassware including petri dishes, Falcon tubes, culture tubes, centrifuge tubes, pipette tips, beakers, filter sterilizers as well as synthetic chemistry glassware and pipettes will cost ($400). HPLC supplies for enzyme assays, pharmacokinetic sample assay shall require solvents ($1,000), chiral and normal and RP columns ($1,200) and UV-vis lamp ($400). We need to dedicate an electrophoresis box ($300) for RNA minigels to avoid contamination and degradation, etc. Electrophoresis supplies including buffers, gels, standards, syringes and maintenance shall cost ($1,000) per year for both protein and DNA work. General laboratory synthetic chemicals, solvents, buffers, reagents and other supplies will cost ($1,000) per year. NMR time at $10/hr will cost ($200). NMR supplies including solvents, chiral shift reagents, tubes and references will cost ($100). A separate set of pipettmen (20, 200 and 1000) will cost ($1,000) per year and are dedicated to RNA extraction and Northern and Southern analysis.

OTHER EXPENSES

Oligonucleotide expenses of primers and probes for Northern and Southern blots will be ($1,500) per year (the UCSF Biomolecular Resource Center synthesizes our oligos for a fee ($35) set up, ($5) per base and ($30) purification or approximately ($300) per probe. We shall synthesize 5 probes.

Mass spectrometry costs associated with operating expenses for obtaining metabolic profiles and pharmacokinetics for infused nicotine studies (GCMS) shall be ($4,000) per year.

Maintenance and repair of HPLC, GC, UV-vis systems necessary for this project shall cost ($800) per year. A 30% UV-vis service contract is ($500), HPLC service is ($60) per hour (parts are extra) and gases and columns for GC shall total ($800) per year.

Duplication and telephone costs for this project will cost ($15) and ($26) per month, respectively for a total of ($500) per year.

Publication costs will include page charges, submission costs, abstract fees and reprint costs. For 2 publications we request ($600) per year.

Office supplies including paper, pens, research notebooks, computer paper and books pertinent to the research proposed shall cost ($200).

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Principal Investigator: John R. Cashman

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
John Richard Cashman	Assistant Professor

EDUCATION (begin with baccalaureate and include postdoctoral training)

Institution and Location	Field of Study	Degree	Year
University of California, Santa Barbara	Chemistry	B.A.	1977
University of California, Santa Barbara	Biology	B.A.	1977
Kansas University, Lawrence, Kansas	Medicinal Chemistry	M.S./Ph.D.	1982
Harvard University, Cambridge, Massachusetts	Bioorganic Chemistry	Postdoc	1984

RESEARCH AND PROFESSIONAL EXPERIENCE (begin with present position; on a separate page, list in chronological order the titles and complete references of all articles published during the last 5 years; list all earlier publications that are pertinent to this application; do not exceed one complete page for publications)

EXPERIENCE

1984-present – Assistant Professor of Chemistry and Pharmaceutical Chemistry, University of California, San Francisco, California

OTHER EXPERIENCE

1985-1988 – Membership Committee, Division of Medicinal Chemistry, American Chemical Society

1986 – Ad Hoc Member, Executive Committee, California State Toxic Substances Research and Training Program

1987 – Committee Member, National Institute of Environmental Health and Science Hazardous Substances Basic Research "Superfund" Program, Phase I

1987 – Committee Member, National Science Foundation "Environmental Chemistry and Chemical Processes" Program

1988 – Committee Member, National Institute of Environmental Health and Science Hazardous Substances Basic Research "Superfund" Program, Phase II

1988,1990 – Committee Member, University Task Force on AIDS, Biological Sciences Study Section

1990 – San Francisco Estuary Project, Technical Advisory Committee

MEMBERSHIP AND AWARDS

REDACTED

PUBLICATIONS (selected from 56)

1. Contribution of N-Oxygenation to the Metabolism of MPTP by Various Liver Preparations. J.R. Cashman and D.M. Ziegler. *Molec. Pharmacol.* 29, 163-167 (1986).
2. Mutagenicity of MPTP (1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine) and its Metabolites. J.R. Cashman. *Toxicology* 43, 173-182 (1987).
3. A Convenient Radiometric Assay for Flavin-Containing Monooxygenase Activity. J.R. Cashman. *Anal. Biochem.* 160, 294-300 (1987).
4. Epoxy Derivatives of Arachidonic Acid are Potent Stimulators of Prolactin Secretion. J.R. Cashman, D. Hanks, and R.L. Weiner. *Neuroendocrinol.* 46, 246-251 (1987).
5. Arachidonate 15-Lipoxygenase from Human Eosinophil-Enriched Leukocytes: Partial Purification of Human Eosinophil 15-Lipoxygenase. E. Sigal, D. Grunberger, J.R. Cashman, C.S. Craik, G.S. Caughey, and J. Nadel. *Biochem. Biophys. Commun.* 150, 376-383 (1988).

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6. Facile N-Oxygenation of MPTP by the Flavin-Containing Monooxygenase. A Convenient Synthesis of Tritiated [^{3}H-CH$_3$]-MPDP$^+$. J.R. Cashman. *J. Med. Chem.* 31, 1258-1261 (1988).
7. Stereoselective N-Oxigenation of Zimeldine and Homozimeldine by the Flavin-Containing Monooxygenase. J.R. Cashman, J. Proudfoot, D.W. Pate, and T. Högberg. *Drug Metab. Dispos.* **16**, 616-622 (1988).
8. Leukotriene Receptor Antagonists. J.R. Cashman and S. Oliva. *Drugs of Today* **24**, 723-732 (1988).
9. Inhibition of Human Leukocyte 5-Lipoxygenase by 15-HPETE and Related Eicosanoids. J.R. Cashman, C. Lambert, and E. Sigal. *Biochem. Biophys. Res. Commun.* **155**, 38-44 (1988).
10. A Reverse-Phase Liquid Chromatographic Assay for Flavin-Containing Monooxygenase Activity. J.R. Cashman and J. Proudfoot. *Anal. Biochem.* **175**, 274-280 (1988).
11. S-Oxygenation of Eptam in Hepatic Microsomes from Fresh- and Saltwater Striped Bass (*Morone saxatillis*). J.R. Cashman, L. Olson, G. Young, and H. Bern. *Chem. Res. Toxicol.* **2**, 392-399 (1989).
12. Chemical and Enzymatic Oxidation of 2-Aryl-1,3-Oxathiolanes: Mechanism of the Hepatic Flavin-Containing Monooxygenase. J.R. Cashman, J. Proudfoot, Y.-K. Ho, M.S. Chin, and L.D. Olsen. *J. Amer. Chem. Soc.* **111**, 4844-4852 (1989).
13. Canrenone Formation *via* General-Base Catalyzed Rearrangement of 7a-(Methylthio)spironolactone S-Oxide. J.R. Cashman ans S. Peña. *Chem. Res. Toxicol.* **2**, 109-113 (1989).
14. 5,6-Epoxyeicosatrienoic Acid Stimulates Growth Hormone Release in Rat Anterior Pituitary Cells. J.R. Cashman. *Life Sciences* **44**(19), 1387-1393 (1989).
15. Eicosanoids Evoke the Release of Amylase and Increase Cytoplasmic Calcium in Rat Parotid Cells. K.W. Snowdowne, L. Rosenoer, E. Yu, and J.R. Cashman. *Biochem. Biophys. Res. Commun.* **161**, 379-384 (1989).
16. Enantioselective N-Oxygenation of Verapamil by the Hepatic Flavin-Containing Monooxygenase. J.R. Cashman. *Molec. Pharmacol.* **36**, 497-503 (1989).
17. S-Oxygenation of 7α-Thiomethylspironolactone by the Flavin-Containing Monooxygenase. J.R. Cashman and S. Peña. *Drug Metabolism and Drug Interactions* **6**, 337-348 (1989).
18. Enantioselective S-Oxygenation of *para* Methoxyphenyl-1,3-dithiolane by Various Tissue Preparations, Effect of Estradiol. J.R. Cashman, L.D. Olsen, C.E. Lambert, and M.J. Presas. *Mol. Pharmacol.* **37**, 319-327 (1990).
19. Stereoselective S-Oxygenation of 2-Aryl-1,3-dithiolanes by Rabbit Lung Preparations. J.R. Cashman and D.E. Williams. *Mol. Pharmacol.* **37**, 333-339 (1990).
20. S-Oxygenation of Dialkyl Sulfides by a Modified Sharpless Reagent: A Model System for the Flavin-Containing Monooxygenase. J.R. Cashman, L.D. Olson, and L. Bornheim. *J. Amer. Chem. Soc.* **112**, 3191-3195 (1990).
21. N-Terminus Determination, FAD and NADP$^+$ Binding Domain Mapping of Hog Liver Flavin-Containing Monooxygenase by Tandem Mass Spectrometry. S. Guan, A. Falick, and J.R. Cashman. *Biochem. Biophys. Res. Commun.* **170**, 937-943 (1990).
22. Enantioselective S-Oxygenation by Hepatic Flavin-Containing and Cytochrome P-450 Monooxygenase. J.R. Cashman, L.D. Olsen, and L. Bornheim. *Chem. Res. Toxicol.* **3**, 344-349 (1990).
23. Stereoselective S-Oxygenation of 2-Aryl-1,3-Dithiolanes by Hog Liver Flavin-Containing Monooxygenase. J.R. Cashman and L.D. Olsen. *Mol. Pharmacol.* **38**, 573-585 (1990).
24. S-Oxygenation of Thiobencarb (Bolero) in Hepatic Preparations from Striped Bass (*Morone saxatillis*) and Mammalian Systems. J.R. Cashman, L.D. Olsen, R.S. Nishioka, E.A. Grey, and H.A. Bern. *Chem. Res. Toxicol.* **3**, 433-440 (1990).
25. Analysis of Amine Metabolites by Ion-Pair High Performance Liquid Chromatography. J.R. Cashman and Z.-C. Yang. *J. Chromatog.* **532**,405-410 (1990).
26. Oxidation of Norzimeldine Hydroxylamine by Hepatic Monooxygenases. J.R. Cashman, Z.-C. Yang, and T. Högberg. *Chem. Res. Toxicol.* **3**, 428-432 (1990).
27. Thioamides. J.R. Cashman. In *Sulfur-Containing Drugs and Related Organic Chemicals: Chemistry, Biochemistry and Toxicology, Vol. 1*, pp. 35-48. Edited by L.A. Damani, Chichester, UK: Ellis Horwood Limited, 1989.
28. Antiinfective Properties of Malathion. R.C. Wester and J.R. Cashman. In *Sulfur-Containing Drugs and Related Organic Chemicals: Chemistry, Biochemistry and Toxicology, Vol. 3*, pp. 181-196. Edited by L.A. Damani, Chichester, UK: Ellis Horwood Limited, 1989.

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Source: https://www.industrydocuments.ucsf.edu/docs/pllm0000

Principal Investigator: John R. Cashman

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Neal L. Benowitz	Associate Professor

EDUCATION (begin with baccalaureate and include postdoctoral training)

Institution and Location	Field of Study	Degree	Year
Rennselaer Polytechnic Institute	Physics	–	–
University of Rochester School of Medicine Rochester, New York	Medicine	M.D.	1969

RESEARCH AND PROFESSIONAL EXPERIENCE (begin with present position; on a separate page, list in chronological order the titles and complete references of all articles published during the last 5 years; list all earlier publications that are pertinent to this application; do not exceed one complete page for publications)

EXPERIENCE

1969-1971	–	Intern and Resident, Internal Medicine, Bronx Municipal Hospital Center, Bronx, New York
1971-1973	–	Fellow in Clinical Pharmacology, Department of Medicine, University of California, San Francisco
1973-1974	–	Clinical Instructor, Department of Medicine, University of California, San Francisco
1975-1976	–	Director, Drug Detoxification Unit, San Francisco General Hospital, San Francisco, California
1979-present	–	Associate Medical Director, San Francisco Bay Area Regional Poison Center, San Francisco, California
1974-present	–	Member of Staff, Medical Service, San Francisco General Hospital, San Francisco, California
1974-1981	–	Assistant Professor in Residence, Departments of Medicine and Psychiatry, University of California, San Francisco
1981-present	–	Associate Professor, Departments of Medicine and Psychiatry, University of California, San Francisco
1983-present	–	Chief, Division of Clinical Pharmacology, University of California, San Francisco

HONORS

REDACTED

PUBLICATIONS (Partial List – Total Publications 126)

1. E.R. Gritz, V. Baer-Weiss, N.L. Benowitz, H. Van Vunakis, and M.E. Jarvik. Plasma Nicotine and Cotinine Concentrations in Habitual Smokeless Tobacco Users. *Clin. Pharmacol. Ther.* **30**, 201-209 (1981).
2. P. Jacob III, M. Wilson, and N.L. Benowitz. Nicotine and Cotinine Determination in Biologic Fluids: An Improved Gas Chromatographic Method. *J. Chromatog.* 222, 61-70 (1981).
3. N.L. Benowitz, F. Kuyt, and P. Jacob III. Circadian Study of Blood Nicotine Concentrations During Cigarette Smoking. *Clin. Pharmacol. Ther.* 32, 758-764 (1982).
4. N.L. Benowitz, P. Jacob III, R.T. Jones, and J. Rosenberg. Interindividual Variability in the Metabolism and Cardiovascular Effects of Nicotine in Man. *J. Pharmacol. Expt. Ther.* **221**, 368-372 (1982).

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5. N.L. Benowitz, S.M. Hall, R.I Herning, P. Jacob III, R.T. Jones, and A.-L. Osman. Smokers of Low Yield Cigarettes Do Not Consume Less Nicotine. *N. Engl. J. Med.* 309, 139-142 (1983).
6. N.L. Benowitz, F. Kuyt, P. Jacob III, R.T. Jones, and A.-L. Osman. Cotinine Disposition and Effects. *Clin. Pharmacol. Ther.* 34, 604-611 (1983).
7. D. Foliart, N.L. Benowitz, and C.E. Becker. Passive Absorption of Nicotine in Flight Attendants (Letter). *New Eng. J. Med.* 308, 1105 (1983).
8. R.I. Herning, R.T. Jones, N.L. Benowitz, and A.H. Mines. How a Cigarette is Smoked Determines Nicotine Blood Levels. *Clin. Pharmacol. Ther.* 33, 84-90 (1983).
9. N.L. Benowitz. The Use of Biologic Fluid Samples in Assessing Tobacco Smoke Consumption. In *Measurement in the Analysis and Treatment of Smoking Behavior.* Edited by J. Grabowski and C.S. Bell. *NIDO Monograph* 48, 6-26 (1984).
10. N.L. Benowitz, F. Kuyt, and P. Jacob III. Influence of Nicotine Delivery on the Cardiovascular and Hormonal Effects of Cigarette Smoking. *Clin. Pharmacol. Ther.* 36, 74-81 (1984).
11. P. Jacob III, C. Savanapridi, L. Yu, M. Wilson, A.T. Shulgin, N.L. Benowitz, B.A. Elias-Baker, S.M. Hall, R.I. Herning, R.T. Jones, and D.P.L. Sachs. Ion-Pair Extraction of Thiocyanate from Plasma and Its Gas Chromatographic Determination Using on-Column Alkylation. *Analyt. Chem.* 56, 1692-1695 (1984).
12. N.L. Benowitz and P. Jacob III. Nicotine and Carbon Monoxide Intake from High- and Low-Yield Cigarettes. *Clin. Pharmacol. Ther.* 36, 265-270 (1984).
13. S.M. Hall, R.I. Herning, R.T. Jones, N.L. Benowitz, and P. Jacob III. Blood Cotinine Levels as Indicators of Smoking Treatment Outcome. *Clin. Pharmacol. Ther.* 35, 810-814 (1984).
14. N.L. Benowitz and P. Jacob III. Daily Intake of Nicotine During Cigarette Smoking. *Clin. Pharmacol. Ther.* 35, 499-504 (1984).
15. Participants: N. Benowitz, M. Feinleib, C. Feyerbend, *et al.* Is There a Future for Lower-Tar-Yield Cigarettes? *Lancet* (November) 16, 1111-1114 (1985).
16. N.L. Benowitz and P. Jacob III. Nicotine Renal Excretion Rate Influences Nicotine Intake During Cigarette Smoking. *J. Pharmacol. Exp. Ther.* 234, 153-155 (1985).
17. R.P. Simon, N. Benowitz, R. Hedlund, and J. Copeland. The Influence of the Blood-Brain pH Gradient on Brain Phenobarbital Uptake During Status Epilepticus. *J. Pharmacol. Exp. Ther.* 234, 830-835 (1985).
18. S.M. Hall, C. Tunstall, D. Rugg, R.T. Jones, and N. Benowitz. Nicotine Gum and Behavioral Treatment in Smoking Cessation. *J. Consult. Clin. Psychol.* 53, 256-258 (1985).
19. S.M. Pond, M.J. Kreek, T.T. Tong, J. Ragunath, and N.L. Benowitz. Altered Methadone Pharmacokinetics in Methadone-Maintained Pregnant Women. *J. Pharmacol. Exp. Ther.* 233, 1-6 (1985).
20. N.L. Benowitz. Clinical Pharmacology of Nicotine. *Annual Review of Medicine, Vol. 37.* Edited by W.P. Creger. Palo Alto, California: Annual Reviews, Inc., 1986, pp. 21-32.
21. N.L. Benowitz, O. Jacob III, L. Yu, and R. Talcott. Reduced Tar, Nicotine and Carbon Monoxide Exposure While Smoking Low and Ultra-Low Yield Cigarettes. *JAMA* 256, 241-246 (1986).
22. P. Jacob III, N.L. Benowitz, L. Yu, and A.T. Shulgin. Determination of Nicotine N-Oxide by Gas Chromatography Following Thermal Conversion to 2-Methyl-6-(3-Pyridyl)-Tetrahydro-1,2-Oxazine. *Analyt. Chem.* 58, 2218-2221 (1986).
23. N.L. Benowitz, P. Jacob III, L. Kozlowski, and L. Yu. Influence of Smoking Fewer Cigarettes on Exposure to Tar, Nicotine, and Carbon Monoxide. *N. Engl. J. Med.* 314, 1310-1313 (1986).
24. N.L. Benowitz, R.T. Jones, and P. Jacob III. Additive Cardiovascular Effects of Nicotine and Ethanol. *Clin. Pharmacol. Ther.* 40, 420-420 (1986).
25. B.L. Lee, N.L. Benowitz, and P. Jacob III. Influence of Tobacco Abstinence on the Disposition Kinetics and Effects of Nicotine. *Clin. Pharmacol. Ther.* 41, 474-478 (1987).
26. N.L. Benowitz, P. Jacob III, and C. Savanapridi. Determinants of Nicotine Intake While Chewing Nicotine Polacrilex Gum. *Clin. Pharmacol. Ther.* 41, 467-473 (1987).
27. P. Jacob III, B.A. Elias-Baker, R.T. Jones, and N.L. Benowitz. Determination of Benzoylecgonine and Cocaine in Biologic Fluids by Automated Gas Chromatography. *J. Chromatog.* (1987).
28. N.L. Benowitz, T. Lake, K.H. Keller, and B.L. Lee. Prolonged Absorption with Development of Tolerance to Toxic Effects Following Cutaneous Exposure to Nicotine. *Clin. Pharmacol Ther.* (1987).
29. C.J. Lynch and N.L. Benowitz. Spontaneous Brand Switching: Consequences for Nicotine and Carbon Monoxide Exposure. *Am. J. Public Health.* (1987).
30. B.L. Lee, N.L. Benowitz, and P. Jacob III. Cigarette Abstinence, Nicotine Gum and Theophylline Disposition. *Ann. Int. Med.* 106, 553-555 (1987).

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Principal Investigator: John R. Cashman

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Sang Bum Park	Postdoctoral Fellow

EDUCATION (begin with baccalaureate and include postdoctoral training)

Institution and Location	Field of Study	Degree	Year
Yonsei University Seoul, Korea	Geochemistry	B.S.	1973
Western Washington University Bellingham, Washington	Chemistry	B.S.	1984
University of Washington Seattle, Washington	Medicinal Chemistry	Ph.D.	1990

RESEARCH AND PROFESSIONAL EXPERIENCE (begin with present position; on a separate page, list in chronological order the titles and complete references of all articles published during the last 5 years; list all earlier publications that are pertinent to this application; do not exceed one complete page for publications)

EXPERIENCE

1990 - present – Postdoctoral Fellow, Department of Pharmaceutical Chemistry, University of California, San Francisco

1985-1990 – Research Assistant, University of Washington, Seattle, Washington

MEMBERSHIPS

REDACTED

PUBLICATIONS

1. G.P. Meier, S.B. Park, G.C. Yee, D.J. Gmur. Isolation, Identification and Semisynthesis of a Unique Human Cyclosporin Metabolite. *Drug Metab. Disp.* 18, 68-71 (1989).
2. G.P. Meier and S.B. Park. A Semi-Synthetic Approach to Olefinic Analogs of Amino Acid One (MeBmt) in Cyclosporin A. *Tetrahedron Lett.* 30, 32-37 (1989).

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Principal Investigator: John R. Cashman

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Peyton Jacob III	Associate Research Chemist

EDUCATION (begin with baccalaureate and include postdoctoral training)

Institution and Location	Field of Study	Degree	Year
University of California, Davis	Chemistry	B.S.	1969
Purdue University, Lafayette, Indiana	Organic Chemistry	Ph.D.	1975
University of California, San Francisco	Pharmaceutical Chemistry	Postdoc	1975-1978

RESEARCH AND PROFESSIONAL EXPERIENCE (begin with present position; on a separate page, list in chronological order the titles and complete references of all articles published during the last 5 years; list all earlier publications that are pertinent to this application; do not exceed one complete page for publications)

EXPERIENCE

1969-1975	–	Teaching and Research Assistant, Department of Chemistry, Purdue University, W. Lafayette, Indiana
1975-1978	–	NIH Postdoctoral Fellow, Department of Pharmaceutical Chemistry, University of California, San Francisco
1978-6/1984	–	Assistant Research Chemist, Departments of Medicine and Psychiatry, University of California, San Francisco, and Division of Clinical Pharmacology, San Francisco General Hospital Medical Center
7/1984-present	–	Associate Research Chemist, Departments of Medicine and Psychiatry, University of California, San Francisco, and Division of Clinical Pharmacology, San Francisco General Hospital Medical Center

HONORS

REDACTED

PUBLICATIONS (Partial List)

1. P. Jacob III, M. Wilson, and N.L. Benowitz. Nicotine and Cotinine Determination in Biologic Fluids: An Improved Gas Chromatographic Method. *J. Chromatog.* 222, 61-70 (1981).
2. P. Jacob III and A.T. Shulgin. The Synthesis of Hydroxybenzaldehydes from Bromobenzaldehydes *via* Lithiated Schiff's Bases. Preparation of 5-Hydroxypiperonal and Related Compounds. *Syn. Commun.* 11, 969-977 (1981).
3. P. Jacob III and A.T. Shulgin. Metallation-Sulfidation: A Convenient Method for the Synthesis or Aryl Alkyl Sulfides and of Unsymmetrical Diaryl Sulfides. *Syn. Commun.* 11, 957-968 (1981).
4. P. Jacob III, J.F. Rigod, S.M. Pond, and N.L. Benowitz. Determination of Methadone and Its Primary Metabolite in Biologic Fluids Using Gas Chromatography with Nitrogen-Phosphorus Detection. *J. Analyt. Toxicol.* 5, 292-295 (1981).
5. P. Jacob III, J.F. Rigod, S.M. Pond, and N.L. Benowitz. Gas Chromatographic Analysis of Meperidine and Normeperidine: Determination in Blood After a Single Dose of Meperidine. *J. Pharmaceut. Sci.* 71, 166-168 (1982).
6. A.T. Shulgin and P. Jacob III. Potential Misrepresentation of 3,4-Methylenedioxyamphetamine (MDA). A Toxicological Warning. *J. Analyt. Toxicol.* 6, 71-75 (1982).

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7. S. Pond, P. Jacob III, M. Humphreys, R. Weiss, and T. Tong. Impaired Metabolism of Methylphenobarbital After a Combined Drug Overdose: Treatment by Resin Hemoperfusion. *J. Toxicol.-Clin. Toxicol.* **19**, 187-196 (1982).
8. A.T. Shulgin and P. Jacob III. 1-(3,4-Methylenedioxyphenyl)-3-Aminobutane: A Potential Toxicological Problem. *J. Toxicol.-Clin. Toxicol.* **19**, 109-110 (1982).
9. N.L. Benowitz, P. Jacob III, R.T. Jones, and J. Rosenberg. Interindividual Variability in the Metabolism and Cardiovascular Effects of Nicotine in Man. *J. Pharmacol. Exp. Ther.* **221**, 368-372 (1982).
10. S.M. Pond, T.G. Tong, N.L. Benowitz, P. Jacob III, and J. Rigod. Lack of Effect of Diazepam on Methadone Metabolism in Methadone-Maintained Addicts. *Clin. Pharmacol. Ther.* **31**, 139-143 (1982).
11. R.P. Simon, N.L. Benowitz, J. Bronstein, and P. Jacob III. Increased Brain Uptake of Lidocaine During Bicuculline-Induced Status Epilepticus in Rats. *Neurology* **32**, 196-199 (1982).
12. P. Jacob III. Resolution of (±)-5-Bromonornicotine. Synthesis of (R)- and (S)-Nornicotine of High Enantiomeric Purity. *J. Organic Chem.* **47**, 4165-4167 (1982).
13. N.L. Benowitz, F. Kuyt, and P. Jacob III. Circadian Blood Nicotine Concentrations During Cigarette Smoking. *Clin. Pharmacol. Ther.* **32**, 758-764 (1982).
14. P. Jacob III and A.T. Shulgin. Sulfur Analogues of Psychotomimetic Agents. 2. Analogs of (2,5-Dimethoxy-4-Methylphenyl)- and (2,5-Dimethoxy-4-Ethylphenyl) Isopropylamine. *J. Med. Chem.* **26**, 746-752, 1983.
15. N.L. Benowitz, F. Kuyt, P. Jacob III, R.T. Jones, and A.-L. Osman. Cotinine Disposition and Effects. *Clin. Pharmacol. Ther.* **34**, 604-611 (1983).
16. N.L. Benowitz, S.M. Hall, R.I. Herning, P. Jacob III, R.T. Jones, and A.-L. Osman. Smokers of Low Yield Cigarettes Do Not Consume Less Nicotine. *New Eng. J. Med.* **309**, 139-142 (1983).
17. N.L. Benowitz and P. Jacob III. Daily Intake of Nicotine During Cigarette Smoking. *Clin. Pharmacol. Ther.* **35**, 499-504 (1984).
18. M.C. Rowbotham, R.T. Jones, N.L. Benowitz, and P. Jacob III. Trazodone-Oral Cocaine Interactions. *Arch. Gen. Psychiat.* **41**, 895-899 (1984).
19. P. Jacob III, B.A. Elias-Baker, R.T. Jones, and N.L. Benowitz. Determination of Cocaine in Plasma by Automated Gas Chromatography. *J. Chromatog.* **306**, 173-181 (1984).
20. N.L. Benowitz and P. Jacob III. Nicotine and Carbon Monoxide Intake While Smoking High- and Low-Yield Cigarettes. *Clin. Pharmacol. Ther.* **36**, 265-270 (1984).
21. N.L. Benowitz, F. Kuyt, and P. Jacob III. Influence of Nicotine Delivery on the Cardiovascular and Hormonal Effects of Cigarette Smoking. *Clin. Pharmacol. Ther.* **46**, 74-81 (1984).
22. S.M. Hall, R.I. Herning, R.T. Jones, N.L. Benowitz, and P. Jacob III. Blood Cotinine Levels as Indicators of Smoking Treatment Outcome. *Clin. Pharmacol. Ther.* **35**, 810-814 (1984).
23. P. Jacob III, C. Savanapridi, L. Yu, M. Wilson, A.T. Shulgin, and N.L. Benowitz. Ion-Pair Extraction of Thiocyanate from Plasma and Its Gas Chromatographic Determination Using On-Column Alkylation. *Analyt. Chem.* **56**, 1692-1695 (1984).
24. D. Lemaire, P. Jacob III, and A.T. Shulgin. Ring-Substituted Beta-Methoxyphenethylamines. A New Class of Psychotomimetic Agents active in Man. *J. Pharm. Pharmacol.* **37**, 575-577 (1985).
25. N.L. Benowitz and P. Jacob III. Nicotine Renal Excretion Rate Influences Nicotine Intake During Cigarette Smoking. *J. Pharmacol. Exp. Ther.* **234**, 153-155 (1985).
26. P. Jacob III, N.L. Benowitz, L. Yu, and A.T. Shulgin. Determination of Nicotine-N-oxide by Gas Chromatography Following Thermal Conversion to 2-Methyl-6-(3-Pyridyl)-Tetrahydro-1,2-Oxazine. *Analyt. Chem.* **58**, 2218-2221 (1986).
27. N.L. Benowitz, R.T. Jones, and P. Jacob III. Additive Cardiovascular Effects of Nicotine and Ethanol. *Clin. Pharmacol. Ther.* **40**, 420-424 (1986).
28. N.L. Benowitz, P. Jacob III, L. Yu, R. Talcott, S. Hall, and R.T. Jones. Reduced Tar, Nicotine, and Carbon Monoxide Exposure While Smoking Ultra-Low But Not Low-Yield Cigarettes. *JAMA* **256**, 241-246 (1986).
29. N.L. Benowitz, P. Jacob III, L.T. Koslowski, and L. Yu. Influence of Smoking Fewer Cigarettes on Exposure to Tar, Nicotine, and Carbon Monoxide. *N. Engl. J. Med.* **315**, 1310-1313 (1986).
30. B.L. Lee, N.L. Benowitz, and P. Jacob III. Influence of Tobacco Abstinence on the Disposition Kinetics and Effects of Nicotine. *Clin. Pharmacol. Ther.* **41**, 474-478 (1987).
31. B.L. Lee, N.L. Benowitz, and P. Jacob III. Cigarette Abstinence, Nicotine Gum and Theophylline Disposition. *Ann. Int. Med.* **106**, 533-555 (1987).
32. N.L. Benowitz, P, Jacob III, and C. Savanapridi. Determinants of Nicotine Intake While Chewing Nicotine Polacrilex gum. *Clin. Pharmacol. Ther.* **41**, 467-473 (1987).

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Principal Investigator: John R. Cashman

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Joëlle Thomas	Postgraduate Researcher

EDUCATION (begin with baccalaureate and include postdoctoral training)

Institution and Location	Field of Study	Degree	Year
University Louis Pasteur Strasbourg, France	Biology	D.E.U.G.	1985
University Louis Pasteur Strasbourg, France	Biochemistry	Licence	1986
University Louis Pasteur Strasbourg, France	Biochemistry Plant Physiology	M.S. M.S.	1987 1987
University Louis Pasteur Strasbourg, France	Molecular and Cellular Biology	D.E.A.	1988

RESEARCH AND PROFESSIONAL EXPERIENCE (begin with present position; on a separate page, list in chronological order the titles and complete references of all articles published during the last 5 years; list all earlier publications that are pertinent to this application; do not exceed one complete page for publications)

EXPERIENCE

1991-present – Postgraduate Researcher, Department of Pharmaceutical Chemistry, University of California, San Francisco

1989-1990 – Research Technician, Appligene, Strasbourg, France

1985-1989 – Research Assistant, University of Louis Pasteur, Strasbourg, France

PUBLICATION

Purification and Reconstitution of the Catalytic Activity of Vegetable Cytochrome P-450: *Trans*-cinnamic Acid 4-Hydroxylase. J. Thomas DEA Thesis.

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OTHER SUPPORT

Provide the details requested concerning all grants, contracts, and applications, active and pending, on which the investigator is a participant. Be sure to include the source of support, the percentage of effort, dates of entire project period, annual direct costs, a brief description of the project, discussion of any overlap or duplication, and any modification that will be made should the present application be funded. If you submit other proposals to the TRDRP, you must list the others as pending support.

B) PENDING SUPPORT CONTINUED:

a) Source: NIH GM 36426 Renewal
Title: Flavin-Containing Monooxygenase: Structural Aspects
% Effort: 20%
Date: 09/01/91 to 08/31/96
Direct Costs: $178,413
Minimal scientific overlap with present submission: purely molecular in nature.

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Principal investigator John R. Cashman

FACILITIES AND RESOURCES

FACILITIES: Mark the facilities to be used at the applicant organization and briefly indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Use "other" to describe the facilities at any other performance sites listed in item 8, page 1, and at sites for field studies. Using continuation pages if necessary, include an explanation of any consortium/contractual arrangements with other organizations.

☒ Laboratory: See Attached

☒ Clinical:

☐ Animal:

☐ Computer:

☐ Office:

☐ Other (_____):

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

See Attached

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretaries, machine shop, and electronics shop, and the extent to which they will be available to the project.

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BIOSAFETY COMMITTEE
Office of Research Affairs, Box 0962
University of California, San Francisco

DATE: March 28, 1990

TO: John R. Cashman, Ph.D.
Pharm. Chem.
Box 0446

FROM: BIOSAFETY COMMITTEE

RE: STATUS OF YOUR BIOSAFETY APPROVAL # R-121-01

In accordance with the Biosafety Committee's responsibility to review Recombinant DNA applications at appropriate intervals, the Committee is checking with all Principal Investigators who were issued permits in 1986.

Please complete this one page questionnaire and return it to: Biosafety Committee, Box 0962. A copy of your approved application is attached. If you have any questions, contact the Biosafety Committee office at 476-2198.

Please check the appropriate line:

___ This protocol is no longer active and there are no plans to reactivate this study in the future.

___ There is a more current approval that includes the research described in the above numbered approval. The approval number of the more current one is _____.

✓ This protocol is currently active. If so, 1) please attach a copy of the current laboratory certification. All labs must be certified at least once every three years. For laboratory certification, contact UCSF Biosafety Officer, at 476-2097. 2) Has the nature of the work changed as to alter any aspect of its biosafety? 2601 X Yes ___ No. If yes, please describe.

Comments Expression of proteins, see enclosure

I certify that I am familiar with the NIH Recombinant DNA Guidelines (enclosed) dated May 7, 1986 and the UCSF Biosafety Manual and agree to observe the safety procedures contained therein.

John Cashman — May 1, 1990
Signature of Principal Investigator — Date

Warren Hensi — 6/8/90
Approved: Chairman, Biosafety Committee — Date

Revised: 3/90

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January 11, 1991

OFFICE OF RESEARCH AFFAIRS
DIVISION OF CONTRACTS AND GRANTS
SAN FRANCISCO, CALIFORNIA 94143-0962
(415) 476-2977

Tobacco-Related Disease Research Program
Office of Health Affairs
Office of the President
University of California
Great Western Building, Suite 500A
2150 Shattuck Avenue
Berkeley, CA 94720

Dear Sir or Madam:

The University of California is presenting for your review a request for support of the following project:

PROJECT TITLE HUMAN MARKERS FOR (S)-NICOTINE BIOTRANSFORMATION AND PATHOLOGY

PRINCIPAL INVESTIGATOR John R. Cashman

TYPE OF PROPOSAL IDEAS

- [X] New
- [ ] Renewal of No.
- [ ] Continuation of No.
- [ ] Supplement to No.
- [ ] Revision of No.

- [X] Grant
- [ ] Contract
- [ ] Fellowship
- [ ] Subcontract
- [ ] Cooperative Agreement

AMOUNT REQUESTED

First Budget Period $75,000
Total Period

INDIRECT COST RATE n/a %

PROJECT PERIOD

First Budget Period 7/1/91 - 6/30/92
Total Period

Your favorable consideration will be appreciated. If you have questions about this proposal please contact Amy J. Ash at (415) 476-8151.

Sincerely,

Lorraine M. Petrakis
Director

cc: Principal Investigator

2023488788

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APPENDICES

John R. Cashman, Ph.D.

"Human Markers for (S)-Nicotine Biotransformation and Pathology"

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Enantioselective S-Oxygenation by Flavin-Containing and Cytochrome P-450 Monooxygenases

John R. Cashman,*,† Leslie D. Olsen,†,‡ and Lester M. Bornheim§

Departments of Pharmaceutical Chemistry and Pharmacology and Liver Center, University of California, San Francisco, California 94143-0446

Received February 19, 1990

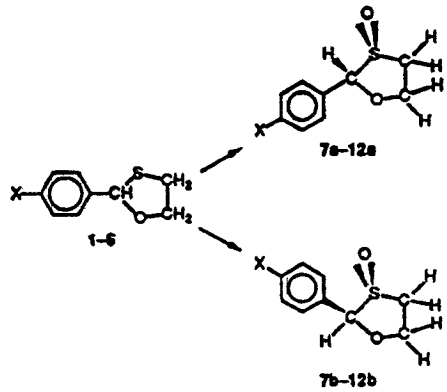
The reaction of the modified Sharpless reagent, as well as microsomes and highly purified flavin-containing monooxygenase from hog liver, and cytochrome P-450IIB-1 from rat liver efficiently S-oxygenates 2-aryl-1,3-oxathiolanes with significant diastereoselectivity and enantioselectivity. The absolute configuration of the synthetic *S*-oxides and the enzyme-derived *S*-oxides was correlated by NMR analysis and by the sign of the Cotton effects obtained from circular dichroism studies. Of the sulfides studied, the *trans-S*-oxide was the major diastereomer produced from monooxygenase-catalyzed biotransformations. In all cases examined, enantioselective S-oxygenation was observed although enantiomeric excess varied from 7 to 100%. In contrast to previous reports, the enantioselectivity of S-oxygenation catalyzed by cytochrome P-450IIB-1 was not always opposite to that of hog liver flavin-containing monooxygenase activity. The presence of the minor *S*-oxide diastereomers in each case was due to incomplete chiral processing by each monooxygenase and not to a competing achiral nonenzymatic process. The results suggest that the active site of hog liver flavin-containing monooxygenase places greater constraints than that of cytochrome P-450IIB-1 on substrate orientation, but in both cases *trans-S*-oxide formation is strongly preferred possibly due to steric interactions of the substrate and the active site.

Introduction

The dialkyl sulfide functionality is present in many important drugs, chemicals, and agrichemicals (*1–3*) and is metabolized principally by S-oxygenation (*4*). In many cases the pharmacodynamics of a drug or chemical has been shown to be dependent on the rate of sulfide oxidation and sulfoxide reduction (*5, 6*). The enzymatic oxidation of sulfides is largely dependent on two microsomal monooxygenases: the cytochromes P-450 (*7*) and the flavin-containing monooxygenase (FMO)[1] (*1, 8–10*). FMO-related S-oxygenations of dialkyl sulfides can be distinguished from cytochrome P-450 related S-oxidations by the use of various inhibitors and pH conditions (*8, 9*) and by the greater susceptibility of hepatic FMO to thermal inactivation (*8*). These two microsomal S-oxygenases may also be distinguished by the stereoselective nature of their enzyme action since it has been reported that FMO and cytochrome P-450 produce *S*-oxides of opposite absolute stereochemistry (*3, 7, 10–12*).

Although less information is known about sulfide oxidation (*13*) than amine oxidation (*14, 15*), cytochrome P-450 oxidation of sulfides has been correlated with the one-electron oxidation potential of the sulfides examined (*16*), and it is possible that sulfides are oxidized by hepatic preparations via single-electron-transfer processes (*17*). In contrast, most of the studies to date indicate that FMO and related hydroperoxyisoalloxazine model systems for FMO operate via nucleophilic anionic mechanisms (*8, 18–20*) although single-electron-transfer mechanisms cannot be ruled out (*9*). In general, it appears that strongly nucleophilic sulfides are substrates for FMO and P-450 while electron-deficient sulfides are substrates for cytochrome P-450 only (*8, 9, 21*). In addition to electronic effects, steric effects may help to determine the monooxygenase that is responsible for the S-oxygenation of sulfides (*8, 12, 22*).

Scheme I. S-Oxygenation of 2-Aryl-1,3-oxathiolanes (1–6) to 2-Aryl-1,3-oxathiolane *S*-Oxides (7a,b–12a,b)[a]



comp no.	X
1, 7	CH_3O
2, 8	CH_3
3, 9	H
4, 10	Cl
5, 11	CN
6, 12	NO_2

[a] Configurational isomers are specified as *trans-S*-oxide (a) and *cis-S*-oxide (b).

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[1] Abbreviations: FMO, flavin-containing monooxygenase; HPLC, high-pressure liquid chromatography; RP-HPLC, reversed-phase high-pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NMR, nuclear magnetic resonance; CD, circular dichroism; ee, enantiomeric excess; de, diastereomeric excess.

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In this study, we determined the diastereoselectivity and enantioselectivity of S-oxygenation of 2-aryl-1,3-oxathiolanes (1–6) catalyzed by purified FMO and microsomes from hog liver as well as purified cytochrome P-450IIB-1 from liver (Scheme I). The S-oxygenation of 2-aryl-1,3-oxathiolanes as stereochemical probes of the active site of FMO and cytochrome P-450 has several advantages, including (1) the formation of stable *cis*- and *trans*-S-oxide diastereomers, which allow for straightforward analysis, and (2) the ease of synthesis of 2-aryl-1,3-oxathiolanes and their *S*-oxides with various electron-rich and electron-deficient aromatic substituents which modulate the relative nucleophilicity of the dialkyl sulfide center. The results of our study provide insight into the stereochemical and mechanisitic details of FMO-catalyzed and cytochrome P-450 catalyzed S-oxygenations.

Experimental Procedures

Materials and Methods. 2-Aryl-1,3-oxathiolanes and their corresponding *S*-oxides were synthesized as previously described (*9, 11*). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, and NADPH were purchased from Sigma Chemical Co. All other reagents and buffers were obtained in the highest quality available from commercial sources. Hog liver microsomes were a generous gift of Professor D. M. Ziegler (University of Texas at Austin), and FMO was isolated and purified from hog liver microsomes by a procedure previously described (*23*). The highly purified hog liver FMO and hog liver microsomes N-oxygenated dimethylaniline very efficiently [545 and 3.4 nmol of product/(min·mg of protein), respectively]. Rat liver cytochrome P-450IIB-1 ($P\text{-}450_{PB}$) (*24*), cytochrome b_5, and NADPH–cytochrome P-450 reductase were purified to homogeneity by a method previously described (*25*).

Rat liver cytochrome P-450IIB-1 was purified as previously described (*26*). Rat liver cytochrome P-450IIB-1 had a specific contents of 19.5 nmol of P-450/mg of protein. The specific content of cytochrome b_5 was 32.8 nmol of b_5/mg of protein, and the reductase preparation had an activity of 66 μmol of cytochrome c reduced/(min·mg of protein) in 0.3 M potassium phosphate buffer (pH 7.7) at 25 °C.

The major phenobarbital-inducible cytochrome P-450 (P-450IIB-1) from rat liver exhibited characteristically high pentoxyresorufin *O*-dealkylase and 16β-testosterone hydroxylase activities [6 and 3 nmol of product/(min·nmol of cytochrome P-450), respectively] shown to be characteristic of the phenobarbital-induced isozymes and was judged to be homogeneous by SDS–polyacrylamide gel electrophoresis (*27*).

The concentration of protein was determined by the method of Bradford (*28*).

Metabolism Incubations and Enzyme Assays. Activity of the microsomal or highly purified FMO was measured by determining S-oxide product formation by HPLC (*29*). The incubation media contained 50 mM potassium phosphate (pH 8.4) and an NADPH-generating system consisting of 0.5 mM $NADP^+$, 2.0 mM glucose 6-phosphate, 1 IU of glucose-6-phosphate dehydrogenase, and 200–300 μg of hog liver microsomes or 40–200 μg of purified hog liver FMO. The reaction was initiated by the addition of substrate and incubated at 33 °C for various times before the reaction was stopped by the addition of 4 volumes of cold CH_2Cl_2 and analyzed for products by HPLC as described below.

Activity of rat liver cytochrome P-450IIB-1 was measured by determining *S*-oxide product formation by HPLC as previously described (*9, 11, 12, 29*). Cytochrome P-450IIB-1 was reconstituted as follows: 50 μg of dilauroylphosphatidylcholine was combined with 600 units of rat cytochrome P-450 reductase and 100 pmol of cytochrome P-450IIB-1. After a 10-min equilibration at 4 °C, the mixture was diluted with potassium phosphate buffer (0.1 M, pH 7.4), substrate was added (400 μM final concentration in 10 μL of methanol), and then 100 pmol of cytochrome b_5 was added. The reaction was immediately initiated by the addition of 1 mM NADPH or a 0.5 mM NADPH-generating system in a final volume of 1 mL. After a 10-min incubation at 33 °C with constant shaking in air the reaction was stopped by the addition of 2 volumes of cold CH_2Cl_2 and prepared for HPLC as previously described (*9, 11, 12, 29*).

Table I. Chiralcel OD HPLC Separation of 2-Aryl-1,3-oxathiolane *S*-Oxides[a]

compound (X group)	retention volume, mL: *trans*-*S*-oxide 1*S*,2*S*	*trans*-*S*-oxide 1*R*,2*R*	*cis*-*S*-oxide 1*S*,2*R*	*cis*-*S*-oxide 1*R*,2*S*
2-aryl-1,3-oxathiolane *S*-oxide				
7 (4-OCH_3)	18.0	12.5	28.6	22.9
8 (4-CH_3)	10.9	8.8	18.3	15.4
9 (4-H)	12.0	9.9	19.7	18.4
10 (4-Cl)	12.0	10.0	17.2	14.4
11 (4-CN)	26.4	23.1	32.8	27.5
12 (4-NO_2)	29.7	24.0	36.5	30.1

[a] HPLC conditions for separation of enantiomers are given under Experimental Procedures.

After analysis of part of the reaction products by RP-HPLC as described previously (*9, 29*), the remaining reaction products were evaporated to dryness and taken up in 2-propanol/hexane (18:82 v/v) for separation and quantitation by chiral HPLC [IBM Model 9000 with UV detector fitted with a Chiracel OD analytical column (25 cm × 0.15 cm i.d. DAICEL Chemical Ind. LTD, New York)] (*11, 12*). The mobile phase was 2-propanol/hexane (18:82 v/v), which efficiently separates the starting material from *cis*- and *trans*-*S*-oxide enantiomers (Table I). Quantification was accomplished by comparing the integrated area for each enantiomer at 240 nm (*9*).

Synthesis of Chiral *S*-Oxides. The synthesis and complete spectral characterization of the 2-aryl-1,3-oxathiolanes 1–6 have been reported previously (*9*). Treatment of compounds 1–6 with sodium metaperiodate resulted in the formation of racemic products although some diastereoselectivity was observed (*30*). The relative stereochemistry of the *S*-oxides was determined by ^1H NMR analysis and is in agreement with reports by others (*31, 32*) which show a downfield shift of signals from hydrogens cis to the sulfoxide oxygen. The synthesis of *S*-oxides with moderate to high enantiomeric excess was accomplished by using a modified Sharpless reagent: titanium isopropoxide–diethyl tartrate in the presence of *tert*-butyl hydroperoxide and water (*33*). The yields and diastereoselectivity of the reactions are similar to those previously reported (*11, 12, 30, 31*). The optical purity of the product *S*-oxides was determined by NMR analysis using a chiral solvating agent [(+)-(*S*)-9-anthryl-2,2,2-trifluoroethanol] for 7–12. The chiral optical properties of the *S*-oxides determined by NMR were confirmed by circular dichroism (CD) studies. The CD absorption spectra were characterized by increasing absorption between 285 and 195 nm with clearly developed peaks and troughs. For the *S*-oxides examined a readily identifiable absorption between 260 and 205 nm corresponds to an absorption band that has information for configuration assignment (*34, 35*). Previous studies have established a relationship between the CD sign and the absolute stereochemistry of the *S*-oxide center (*36*) where a positive CD can be correlated with an (*R*)-sulfoxide configuration. Accordingly, we correlated the absolute configuration of the *S*-oxides obtained by chemical synthesis. The stereochemistry of the second (benzylic) center was defined by the geometric requirement of the cis or trans configuration as determined by NMR spectroscopy. HPLC of the enantiomers with a Chiralcel OD chiral column confirmed the enantiomeric excess (ee) determined by NMR and CD. The retention volumes of all four enantiomers for each 2-aryl-1,3-oxathiolane *S*-oxide (7–12) are listed in Table I. By use of this HPLC system, efficient separation of the enantiomers was obtained and ee could be readily determined. In the event that trace impurities interferred with the HPLC runs, the *S*-oxide metabolites were first purified with a RP-HPLC system, extracted with CH_2Cl_2, dried, and reanalyzed by the DAICEL HPLC system.

Results

S-Oxygenation of 2-Aryl-1,3-oxathiolanes by Hog Liver Microsomes and Hog Liver FMO. We investigated the chemical and enzymatic S-oxygenation of 2-aryl-1,3-oxathiolanes (1–6) in vitro (Scheme I). The S-

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Table II. S-Oxygenation of 2-Aryl-1,3-oxathiolanes with Hog Liver Microsomes

substrate[a] (X group)	% de[b]	% ee[c] trans-S-oxide 1S,2S	1R,2R	cis-S-oxide 1S,2R	1R,2S
+n-octylamine					
1 (4-OCH_3)	46.3 ± 5.1	97.3 ± 1.0		99.1 ± 1.5	
2 (4-CH_3)	22.4 ± 16.8	100		100	
3 (4-H)	15.9 ± 1.6	100		100	
4 (4-Cl)	28.2 ± 6.8	97.9 ± 1.8		100	
5 (4-CN)	65.9 ± 3.8	97.5 ± 3.1		100	
6 (4-NO_2)	40.9 ± 0.4	92.0 ± 2.3		100	
−n-octylamine					
1 (4-OCH_3)	77.0 ± 5.1	80.9 ± 2.0		50.7 ± 1.6	
2 (4-CH_3)	60.5 ± 1.7	79.7 ± 0.6		100	
3 (4-H)	28.8 ± 2.3	54.6 ± 5.1		100	
4 (4-Cl)	70.8 ± 2.1	71.3 ± 1.1		100	
5 (4-CN)	100	81.4 ± 1.1			
6 (4-NO_2)	98.1 ± 0.8	74.1 ± 0.1			100

[a] The complete incubation system contained 50 mM phosphate buffer (pH 8.4), 200 μM substrate, the NADPH-generating system, 875 μg of microsomal protein, and 5 mM n-octylamine in a final volume of 0.25 mL. The results are the average of three or four determinations (±SD). [b] Percent diastereomeric excess (de) refers to, for example, percent trans-S-oxide (% 1R,2R + % 1S,2S) − percent cis-S-oxide (% 1R,1S + % 1S,2R). For 1–6 de refers to trans-S-oxide. [c] Percent enantiomeric excess (ee) for each diastereomer refers to the percent enantiomer in excess subtracted from the percent of the minor enantiomer divided by the total percent of enantiomers in question. This value is multiplied by 100.

Table III. S-Oxygenation of 2-Aryl-1,3-oxathiolanes with Hog Liver Flavin-Containing Monooxygenase

substrate[a] (X group)	% de[b]	% ee[c] trans-S-oxide 1S,2S	1R,2R	cis-S-oxide 1S,2R	1R,2S
+n-octylamine					
1 (4-OCH_3)	31.6 ± 5.5	49.1 ± 6.7		53.4 ± 6.7	
2 (4-CH_3)	23.6 ± 2.0	86.3 ± 3.4		100	
3 (4-H)	6.5 ± 2.6	100		100	
4 (4-Cl)	20.0 ± 3.9	84.8 ± 2.5		100	
5 (4-CN)	69.9 ± 23.3	92.9 ± 2.1		100	
6 (4-NO_2)	32.7 ± 1.9	81.4 ± 2.2		100	
−n-octylamine					
1 (4-OCH_3)	29.4 ± 0.3	85.9 ± 4.1		100	
2 (4-CH_3)	19.3 ± 1.0	88.2 ± 3.6		100	
3 (4-H)	9.2 ± 1.5	100		100	
4 (4-Cl)	6.9 ± 2.7	87.2 ± 1.9		100	
5 (4-CN)	58.1 ± 2.2	94.9 ± 2.4		100	
6 (4-NO_2)	42.9 ± 2.2	83.5 ± 1.7		100	

[a] The complete system is as described in Table II except 225 μg of highly purified FMO protein was used. The results are the average of three or four determinations (±SD). [b] Diastereomeric excess (de) as defined in Table II. The de refers to trans-S-oxide. [c] Enantiomeric excess (ee) as defined in Table II.

oxidative biotransformation of 2-aryl-1,3-oxathiolanes was determined with hog liver microsomes and purified hog liver FMO. As shown by previous studies, mono- and disulfoxide products were obtained from hepatic microsome or purified monooxygenase preparations (*9, 29*). The relative rate of hog liver microsomes and FMO-catalyzed *S*-oxide formation was quite similar to the rate of *S*-oxide formation previously reported for this series of compounds (*9, 29*). During the short incubation times used for the present study, only *S*-monooxides were obtained. As shown in Table II, 2-aryl-1,3-oxathiolanes were S-oxygenated mainly to produce *trans-S*-oxides by hog liver microsomes. In the absence of *n*-octylamine, a positive effector for hepatic FMO (*8*), and an inhibitor of cytochrome P-450 (*37*), a marked increase in the S-oxygenation diastereoselectivity as compared to the complete microsomal system and a significant decrease in the enantiomeric enrichment for *pro-S* S-oxygenation were observed. In the presence of highly purified hog liver FMO, S-oxygenation of 2-aryl-1,3-oxathiolanes produces the *trans-S*-oxide isomer with significant diastereoselectivity, and in the absence of *n*-octylamine a slight increase in enantiomeric excess of the *trans*-(1*S*,2*S*)-*S*-oxide was observed (Table III). For both the microsomal and highly purified hog liver FMO a marked stereopreference for formation of *trans*-(1*S*,2*S*)-*S*-oxide or the *cis*-(1*S*,2*R*)-*S*-oxide was observed. Thus, for 2-aryl-1,3-oxathiolanes, preference for addition of an oxygen to the *pro-S* sulfur atom lone pair was seen.

S-Oxygenation of 2-Aryl-1,3-oxathiolanes by Cytochrome P-450IIB-1. The S-oxygenation of sulfides 1–6 was investigated with purified cytochrome P-450IIB-1 in order to determine the rate of *S*-oxide formation as well as the diastereoselectivity and enantioselectivity of the reaction. 2-Aryl-1,3-oxathiolanes are good substrates for rat liver cytochrome P-450IIB-1. In the presence of purified cytochrome P-450IIB-1 from rat liver, S-oxygenation of 2-aryl-1,3-oxathiolanes 1–6 gave predominantly the *trans-S*-oxide diastereomer. As shown in Table IV, S-oxygenation of sulfides 1–6 gave a major *trans-S*-oxide product, but generally (i.e., with the exception of 5) only a modest excess of one enantiomer was observed to be formed. For rat liver cytochrome P-450IIB-1, the stereopreference for oxygenation at sulfur indicates that attack of an oxygen atom at the *pro-S* sulfur atom occurred for aryl-1,3-oxathiolanes 1–3 and 6. For compounds 4 and 5 a stereopreference for oxygenation of the sulfur atom at *pro-R* sulfur was observed. As shown in Table IV, the rates

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Table IV. S-Oxygenation of 2-Aryl-1,3-oxathiolanes by Rat Liver Cytochrome P-450IIB-1

	S-oxide formed, nmol/(min·nmol of P-450)			% ee[c]			
				trans-S-oxide		cis-S-oxide	
substrate[a] (X group)	cis-S-oxide	trans-S-oxide	% de[b]	1R,2R	1S,2S	1R,2S	1S,2R
1 (4-OCH_3)	0.2	4.9	92.0		11.0		7.0
2 (4-CH_3)	0.1	0.9	80.0		6.9		20.6
3 (4-H)	0.1	0.3	75.0		8.3		24.9
4 (4-Cl)	0.1	2.1	90.0	7.1			7.8
5 (4-CN)	2.5	8.3	53.9	100		100	
6 (4-NO_2)	1.2	8.8	76.0		9.9	9.4	

[a]The incubation system contained 50 mM phosphate, pH 7.4, the NADPH-generating system, 400 μM substrate, 50 μM dilauroylphosphatidylcholine, 600 units of cytochrome c reductase, 100 pmol of cytochrome b_5, and 100 pmol of cytochrome P-450IIB-1, and values are the average of two or three determinations. [b]Percent diastereomeric excess (de) as defined in Table II. [c]Enantiomer excess (ee) as defined in Table II.

of S-oxide formation did not follow a strict Hammett-type dependence (*38*) on the nature of the aryl substituent, but there was a tendency for electron-deficient 2-aryl-1,3-oxathiolanes 4–6 to be oxidized by cytochrome P-450IIB-1 to a greater extent than electron-rich 2-aryl-1,3-oxathiolanes 2 and 3. In each case examined, the *trans*-S-oxide product had an excess of one enantiomer. The stereochemistry of S-oxygenation by rat liver cytochrome P-450IIB-1 was determined and indicated a preference for formation of the (1S,2S)-*trans*-S-oxide (Table IV) except for compounds 4 and 5.

Discussion

Efficient methods for the synthesis and analysis of enantiomers of 2-aryl-1,3-oxathiolane S-oxides (7–12) have been developed (*30, 32, 33*) and used to investigate the stereoselective S-oxygenation catalyzed by hog liver microsomes, hog liver FMO, and rat liver cytochrome P-450IIB-1 (Scheme I). 2-Aryl-1,3-oxathiolanes are good substrates for each monooxygenase enzyme system examined (*9, 29, 30*) and provide a convenient tool to study active site topology of both monooxygenase systems.

In microsomal preparations from hog liver the major product of metabolism of 2-aryl-1,3-oxathiolanes 1–6 is the *trans*-S-oxide. The diastereoselective S-oxygenation of 1–6 reported here is less than or in some cases the same as that reported previously (*9*). We attribute the differences to the nature of the composition and activity of the microsomal monooxygenase in this preparation of microsomes. In the absence of *n*-octylamine, the diastereoselectivity of microsomal S-oxygenation is increased for all cases tested (Table II), but surprisingly, the enantioselectivity is slightly decreased. This may be a consequence of the contribution of cytochromes P-450 to the enantioselective S-oxygenation of 1–6. In contrast, for incubations performed with highly purified hog liver FMO in the presence or absence of *n*-ocytlamine, no significant difference in the diastereoselectivity or enantioselectivity is apparent except for compound 1 (Table II). Thus, for 1, preference for addition of an oxygen atom to the *pro-S* sulfur atom by hog liver microsomes in the presence of *n*-octylamine was observed to be 98.8% (i.e., 70.2% 1S,2S; 28.6% 1S,2R) with 1.0% addition at the *pro-R* sulfur atom (i.e., 0.6% 1R,2R; 0.4% 1R,2S). While the diastereoselectivity of S-oxygenation catalyzed by highly purified hog liver FMO is reported here as decreased compared to our previous report (*9*), nevertheless, addition of molecular oxygen to the *pro-S* sulfur atom is clearly the predominant pathway for the hog liver FMO. S-Oxygenation of 1–6 by rat liver cytochrome P-450IIB-1 is highly diastereoselective but, in general, only modestly enantioselective except for 5 (Table IV). For example, rat liver cytochrome P-450IIB-1 S-oxygenates 1 with preference for addition of oxygen to the *pro-S* sulfur atom to be 53.7% (i.e., 51.7% 1S,2R; 2.0% 1S,2S) with 46.3% addition of oxygen to the *pro-R* sulfur atom (i.e., 44.7% 1R,2S; 1.6% 1R,2R). From these results we conclude that hog liver FMO possesses significant active site constraints and requires rigid orientation of substrate whereas cytochrome P-450IIB-1 possesses a great deal more flexibility at the active site. For FMO, there is clear steric interference to the aryl group and *cis*-S-oxide formation. A possible explanation for the high enantioselectivity for FMO-catalyzed S-oxygenation and lower enantioselectivity for cytochrome P-450IIB-1 catalyzed S-oxygenation may be due to the involvement of anionic nucleophilic mechanisms in the former, and radical species involvement in the latter case (*9*). From the data for S-oxygenation of 2-aryl-1,3-oxathiolanes by cytochrome P-450IIB-1, a clear distinction for single-electron transfer versus a nucleophilic mechanism cannot be made (*39*), but the data are consistent with a role of single-electron transfer in the product-determining step of the reaction (*16, 17*). For FMO, a nucleophilic mechanism is favored, but rapid collapse of radical species to produce S-oxide products cannot be excluded.

Analysis of electronic substituent effects on hog liver microsomes or rat liver cytochrome P-450IIB-1 catalyzed S-oxygenations of 1–6 reveals a nonlinear Hammett-type substituent correlation (*38*). Such "U-shaped" correlations may suggest that possibly two reaction mechanisms are operating, with one type of mechanism taking place with electron-rich sulfide substrates and a different one with electron-deficient sulfide substrates. Another possibility is that the rate-limiting step for S-oxygenation is different for electron-deficient and electron-rich sulfides. That a linear correlation for the S-oxygenation of 1–6 (*9*) was obtained with highly purified FMO suggests that hog liver cytochrome P-450 may contribute to S-oxygenation by a different mechanism possibly involving nonnucleophilic reactions. Evidence for single-electron-transfer mechanisms by the cytochrome P-450IIB-1 catalyzed S-oxygenations is obtained from studies with compounds 1–4 where very low enantioselectivity is observed. While relatively high enantioselectivity for S-oxygenation of 5 by both hog liver FMO and rat liver cytochrome P-450IIB-1 is observed, a clear distinction in mechanism cannot be made. Such data are consistent with an anionic nucleophilic mechanism of S-oxygenation for FMO, but they do not rule out the involvement of single-electron-transfer mechanisms (*9*). It is possible that, for cytochrome P-450IIB-1, slightly different mechanisms of oxidation are at work for different classes of sulfides as well as for sulfides within a series.

In general, cytochrome P-450IIB-1 catalyzes S-oxygenations of 2-aryl-1,3-oxathiolanes with less diastereomeric precision than hog liver FMO. This has also been observed for highly purified and microsomal pulmonary flavin-containing and cytochrome P-450 monooxygenases

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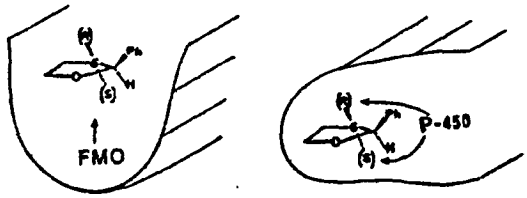

Figure 1. Stereoselective S-oxygenation of 2-aryl-1,3-oxathiolanes by hog liver flavin-containing monooxygenase and cytochrome P-450IIB-1.

from pregnant rabbit (*12*). For hog liver FMO significant active site steric constraints must be at work in order to effect such remarkable diastereoselectivity as that observed in this study. In agreement with previous observations concerning heteroatom oxygenations (*21, 40, 41*), hog liver FMO accepts substrates to afford *trans-S*-oxide products. As shown in Figure 1, FMO enzymatic steric bulk must almost preclude attack from the cis face of the 2-aryl-1,3-oxathiolane substrate. With the relative configuration of the substrate fixed, as shown in Figure 1, attack of oxygen by FMO or cytochrome P-450IIB-1 must occur as depicted to give *S*-oxide of the absolute stereochemistry determined. It is currently unclear as to what subtle enzymic features direct attack of oxygen at the *pro-S* or *pro-R* position of the sulfide molecule, but cytochrome P-450 appears capable of an "edge-on" mode of S-oxygenation, whereas for FMO, orientation of the entire molecule in a binding pocket is required for S-oxygenation. The results described above are consistent with the view (*42*) that FMO involves anionic nucleophilic attack of sulfide sulfur on electrophilic oxygen and that cytochrome P-450 catalyzed S-oxygenation is initiated by a single-electron transfer from sulfide sulfur.

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References

(1) Ziegler, D. M. (1982) Functional groups bearing sulfur. In *Metabolic basis of detoxication* (Jacoby, W. B., Bend, J. R., and Caldwell, J., Eds.) pp 171–184, Academic Press, New York.
(2) Mitchell, S. C., and Waring, R. H. (1986) The early history of xenobiotic sulfoxidation. *Drug Metab. Rev.* 16, 255–284.
(3) Levi, P. E., and Hodgson, E. (1988) Stereospecificity in the oxidation of phorate and phorate sulfoxide by purified FAD-containing monooxygenase and cytochrome P-450 isozymes *Xenobiotica* 18, 29–39.
(4) Damani, L. A. (1987) in *Drug Metabolism—From Microbes to Man* (Benford, D. J. Bridges, J. W., and Gibson, G. G., Eds.) pp 581–603, Taylor and Francis, London.
(5) Duggan, D. E. (1981) Sulindac: therapeutic implications of the prodrug/pharmacophore equilibrium. *Drug Metab. Rev.* 12, 325–337.
(6) Renwick, A. G. (1989) Sulfoxides and sulfones. In *Sulfur-containing drugs and related organic compounds* (Damani, L. A., Ed.) Vol. 1, Part B, pp 133–154, Ellis Horwood, Chichester.
(7) Waxman, D. J., Light, D. R., and Walsh, C. T. (1982) Chiral sulfoxidations catalyzed by rat liver cytochromes P-450. *Biochemistry* 21, 2499–2507.
(8) Ziegler, D. M. (1980) Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds. In *Enzymatic Basis of Detoxication* (Jacoby, W. B., Ed.) pp 201–227, Academic Press, New York.
(9) Cashman, J. R., Proudfoot, J., Ho, Y.-K., Chin, M. S., and Olsen, L. D. (1989) Chemical and enzymatic oxidation of 2-aryl-1,3-oxathiolanes: mechanism of the hepatic flavin-containing monooxygenase. *J. Am. Chem. Soc.* 111, 4844–4852.
(10) Light, D. R., Waxman, D. J., and Walsh, C. T. Studies on the chirality of sulfoxidation catalyzed by bacterial flavoenzyme cyclohexanone monooxygenase and hog liver flavin adenine dinucleotide containing monooxygenase. *Biochemistry* 21, 2490–2498.
(11) Cashman, J. R., Olsen, L. D., Lambert, C. E., and Presas, M. J. (1990). Enantioselective S-oxygenation of *para*-methoxy-phenyl-1,3-dithiolane by various tissue preparations: effect of estradiol. *Mol. Pharmacol.* 37, 319–327.
(12) Cashman, J. R., and Williams, D. E. (1990). Enantioselective S-oxygenation of 2-aryl-1,3-dithiolanes by rabbit lung enzyme preparations. *Mol. Pharmacol.* 37, 333–339.
(13) Damani, L. A. (1989) Thioethers, thiols, dithioic acids and disulfides: Phase I reactions. In *Sulfur Containing Drugs and Related Organic Compounds* (Damani, L. A., Ed.) Vol. 1, Part A, pp 135–150, Ellis Horwood, Chichester.
(14) Tullman, R. H., and Hanzlik, R. P. (1984) Inactivation of cytochrome P-450 and monoamine oxidase by cyclopropylamines. *Drug Metab. Rev.* 15, 1163–1182.
(15) Guengerich, F. P., and MacDonald, T. L. (1984) Chemical mechanism of catalysis by cytochrome P-450: A unified view. *Acc. Chem. Res.* 17, 9–16.
(16) Watanabe, Y., Numata, T., Iyanagi, T., and Ose, S. Enzymatic oxidation of alkyl sulfides by cytochrome P-450 and hydroxyl radical. *Bull. Chem. Soc. Jpn.* 54, 1163–1170.
(17) Watanabe, Y., Iyanagi, T., and Ose, S. Kinetic study on enzymatic S-oxygenation promoted by a reconstituted system with purified cytochrome P-450. *Tetrahedron Lett.* 21, 3685–3688.
(18) Ball, S., and Bruice, T. C. Oxidation of amines by a 4a-hydroperoxyflavin. *J. Am. Chem. Soc.* 101, 4017–4019.
(19) Miller, A. (1982) A model for FAD-containing monooxygenase: the oxidation of thioanisole derivatives by an isoalloxazine hydroperoxide. *Tetrahedron Lett.* 23, 753–757.
(20) Doerge, D. R., and Corbett, M. D. (1984) Hydroperoxyflavin-mediated oxidations of organosulfur compounds: Model studies for the flavin monooxygenase. *Mol. Pharmacol.* 26, 348–352.
(21) Damani, L. A., and Houdi, H. H. (1988) Cytochrome P-450 and FAD-monooxygenase mediated S- and N-oxygenations. *Drug Metab. Drug Interact.* 6, 350–363.
(22) Hajjar, N. P., and Hodgson, E. (1980) Flavin-adenine dinucleotide-dependent monooxygenase: Its role in the sulfoxidation of pesticides in mammals. *Science* 209, 1134–1136.
(23) Sabourin, P. J., Smyser, B. P., and Hodgson, E. (1984) Purification of the flavin-containing monooxygenase from mouse and pig liver microsomes. *Int. J. Biochem.* 16, 713–720.
(24) Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, E. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Philips, I. R., Sato, R., and Waterman, M. R. (1989) The P-450 superfamily: Updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* 8, 1–13.
(25) Waxman, D. J., and Walsh, C. (1982) Phenobarbital-induced rat liver cytochrome P-450. *J. Biol. Chem.* 257, 10446–10457.
(26) Bornheim, L. M., and Correia, M. A. (1989) Purification and characterization of a mouse liver cytochrome P-450 induced by cannabidiol. *Mol. Pharmacol.* 36, 377–383.
(27) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227, 680–685.
(28) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
(29) Cashman, J. R., and Proudfoot, J. (1989) A reverse-phase high pressure liquid chromatographic assay for flavin-containing monooxygenase activity. *Anal. Biochem.* 175, 274–280.
(30) Cashman, J. R., Olsen, L. D., and Bornheim, L. M. (1990) Oxygenation of dialkylsulfides by a modified sharpless reagent: A model system for the flavin-containing monooxygenase. *J. Am. Chem. Soc.* 112, 3191–3195.
(31) Holland, H. L., and Munoz, B. (1988) Fungal biotransformation of 1,3-oxathiolanes. *Can. J. Chem.* 66, 2299–2303.
(32) Bortolini, O., DiFuria, F., Licini, G., Modena, G., and Rossi, M. (1986) Asymmetric oxidation of 1,3-dithiolanes. A route to the optical resolution of carbonyl compounds. *Tetrahedron Lett.* 27, 6257–6260.
(33) Pitchen, P. E., Dunach, E., Desmukh, M. N., and Kagan, H. B. (1984) An efficient asymmetric oxidation of sulfides to sulfoxides. *J. Am. Chem. Soc.* 106, 8188–8193.

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(34) Auret, B. J., Boyd, D. R., Breen, F., Greene, R. M. E., and Robinson, P. M. (1981) Stereoselective enzyme-catalyzed oxidation–reduction reactions of thioacetals–thioacetal S-oxides by fungi. *J. Chem. Soc., Perkin Trans. 1*, 930–933.

(35) Auret, B. J., Boyd, D. R., Cassidy, E. S., Hamilton, R., Turley, F., and Drake, A. F. (1985) Structure and absolute stereochemistry of thioacetal S-oxides obtained by fungal metabolism of 2-alkyl-1,3-dithianes. *J. Chem. Soc., Perkin Trans. 1*, 1547–1552.

(36) Mislow, K. M., Green, M., Laur, P., Meilillo, J. T., Simmons, T., and Ternay, A. L., Jr. (1965) Absolute configuration and optical rotary power of sulfoxides and sulfinate esters. *J. Am. Chem. Soc.* 87, 1958–1976.

(37) Jefcoate, C. R. E., Gaylor, J. L., and Calabrese, R. L. (1969) Ligand interactions with cytochrome P-450: binding of primary amines. *Biochemistry* 8, 3455–3465.

(38) Lowry, R. H., and Richardson, K. S. (1981) *Mechanism and Theory in Organic Chemistry*, 2nd ed. pp 130–142, Harper Row, New York.

(39) Pryor, W. A., and Hendrickson, W. H. (1983) Reaction of nucleophiles with electron acceptors by S_N2 or electron transfer (ET) mechanisms: tert-butylperoxybenzoate/dimethylsulfide and benzoylperoxide/N,N-dimethylaniline systems. *J. Am. Chem. Soc.* 105, 7114–7122.

(40) Williams, D. E., Shigenaga, M. K., and Castagnoli, N., Jr. (1990) The role of cytochromes P-450 and flavin-containing monooxygenase in the metabolism of (S)-nicotine by rabbit lung. *Drug Metab. Dispos.* (in press).

(41) Damani, L. A., Pool, W. F., Crooks, P. A., Kaderlik, R. K., and Ziegler, D. M. (1988) Stereoselectivity in the N'-oxidation of nicotine isomers by flavin-containing monooxygenase. *Mol. Pharmacol.* 33, 702–705.

(42) Oae, S., Mikami, A., Matsura, T., Ogawa-Asada, K., Watanabe, Y., Fujimori, K., and Iyanagi, T. (1985) Comparison of sulfide oxygenation mechanism for liver microsomal FAD-containing monooxygenase with that for cytochrome P-450. *Biochem. Biophys. Res. Commun.* 131, 567–573.

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Enantioselective S-Oxygenation of 2-Aryl-1,3-dithiolanes by Rabbit Lung Enzyme Preparations

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SUMMARY

Pulmonary microsomes, highly purified pulmonary flavin-containing monooxygenase, and highly purified pulmonary cytochrome P-450$_{m-4}$ from pregnant female rabbits catalyze the NADPH-dependent *S*-oxygenation of a series of 2-aryl-1,3-dithiolanes. The *S*-oxide is the only detectable product formed during the short time period of the enzymatic reactions. Studies on the biochemical mechanism for *S*-oxygenation of 2-aryl-1,3-dithiolanes suggest that this reaction is catalyzed preferentially by the flavin-containing monooxygenase, although cytochromes P-450 also contribute to *S*-oxygenation. This conclusion is based on the effects of a cytochrome P-450 inhibitor, aminobenzotriazole, as well as on studies of the stereoselectivity of the reaction. Although both purified rabbit pulmonary cytochrome P-450$_{m-4}$ and purified flavin-containing monooxygenase have identical diastereoselectivity, producing the (*trans*)-*S*-oxide, these monooxygenases possess opposite *S*-oxygenation enantioselectivity. Pulmonary cytochrome P-450$_{m-4}$ *S*-oxygenates 2-aryl-1,3-dithiolanes almost exclusively at the *pro-S*-sulfur atom, whereas pulmonary flavin-containing monooxygenase *S*-oxygenates 2-aryl-1,3-dithiolanes exclusively at the *pro-R*-sulfur atom. 2-Aryl-1,3-dithiolane *S*-oxides are *S*-oxygenated a second time on the *S'*-sulfide sulfur atom but only by rabbit lung microsomes and pulmonary flavin-containing monooxygenase and not by cytochrome P-450$_{m-4}$. That pulmonary flavin-containing monooxygenase only catalyzes formation of (*trans*)- and not (*cis*)-2-aryl-1,3-dithiolane *S*-oxide formation suggests that the active site of pulmonary flavin-containing monooxygenase exerts great steric limitations on 2-aryl-1,3-dithiolane *S*-oxygenation.

The dialkylsulfide (or thioether) group occurs widely in drugs, chemicals, and pesticides (1). Among the routes of metabolism available to dialkylsulfides are oxidation to the sulfoxide or *S*-dealkylation. *S*-Dealkylation is a minor route of biotransformation (2–4). Once formed, dialkylsulfide *S*-oxides may be further oxidized to dialkylsulfones or, alternatively, reduced to the parent sulfide. Several examples of important sulfide-containing drugs or chemicals metabolized by these routes both *in vitro* and *in vivo* have been described (5–8).

Dialkylsulfoxide metabolites are generally chemically stable to hydrolysis, rearrangement, or racemization, although elimination reactions are possible (9). Sulfone metabolites are, likewise, generally chemically stable, although the amount of sulfone formed *in vivo* from the sulfide is usually substantially less than the amount of sulfoxide. This may be a consequence of the relative hydrophilicity of the sulfoxide metabolite or the relative nucleophilicity of the sulfoxide sulfur, compared with sulfide sulfur, towards *S*-oxygenation. A decrease in the relative hydrophilicity and nucleophilicity of the sulfur atom, as seen in the case of sulfoxide sulfur, generally results in a decrease in monooxygenase-catalyzed *S*-oxygenation (8, 10). Reductive metabolic processes may also help determine the amount of sulfone formed, because sulfoxides are easily reduced whereas sulfones are not reduced at all (4). The three monooxygenase systems primarily involved in dialkylsulfide *S*-oxygenation are cytochrome P-450, flavin-containing monooxygenase, and prostaglandin synthetase. Generally, highly nucleophilic dialkylsulfide sulfur atoms are *S*-oxygenated by the flavin-containing monooxygenase (8, 11, 12), prostaglandin synthetase (13), and cytochrome P-450, whereas nonnucleophilic sulfur atoms are also *S*-oxygenated by cytochrome P-450 (14, 15). The relative contribution to the *S*-oxygenation of a dialkylsulfide by each monooxygenase has been investigated in only a few cases (11, 15–18).[1]

Recently, another form of the flavin-containing monooxygenase was isolated from rabbit lung and found to be immunochemically and catalytically distinct from the liver enzyme (19, 20), in agreement with earlier studies that suggested that the lung possessed some unusual metabolic properties (21). In

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[1] J. R. Cashman, L. D. Olsen, C. E. Lambert, and M. J. Presas, Enantioselective *S*-oxygenation of *para*-methoxy-phenyl-1,3-dithiolane by various tissue preparations: effect of estradiol. Submitted for publication.

ABBREVIATION: HPLC, high pressure liquid chromatography.

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contrast to hepatic systems (12, 15–17),[1] to date, the enzymatic basis for the studies of the stereoselective *S*-oxygenation of sulfides in rabbit lung has not been reported. The purpose of this investigation, therefore, was to determine the stereoselective *S*-oxygenation of a model sulfide with rabbit lung microsomes and compare these results with the major purified *S*-monooxygenases present in lung microsomes from pregnant rabbits, namely pulmonary flavin-containing monooxygenase and pulmonary cytochrome P-450_{IIB-4}. The *para*-substituted aryl-1,3-dithiolanes were employed in this study because they possess several features useful for investigation of *S*-oxygenase activity; (a) *para*-substituted aryl-1,3-dithiolanes are good substrates for both purified cytochrome P-450_{IIB-4} and purified flavin-containing monooxygenase, (b) enzymatic *S*-oxygenation of 1–5 may occur with diastereotopic selectivity and may also result in enantioenrichment of an *S*-oxide product by selection between two enantiotopic sulfur atom lone pairs that are stable to pyramidal inversion, and (c) during the short incubation times used in these studies, the aryl-1,3-dithiolane *S*-oxides formed did not undergo further *S*-oxygenation, reduction, or decomposition to other products.

In this report we determine the diastereoselectivity and enantioselectivity of *S*-oxygenation of 2-aryl-1,3-dithiolanes catalyzed by microsomes and highly purified flavin-containing monooxygenase and cytochrome P-450_{IIB-4} from female rabbit lung. In addition, we examine the *S*-oxygenation of 2-aryl-1,3-dithiane *S*-oxides. The results of our study demonstrate the utility of investigating cytochrome P-450- and flavin-containing monooxygenase-catalyzed *S*-oxygenations of aryl-1,3-dithiolanes as mechanistic and stereochemical probes of enzyme function.

Experimental Procedures

Methods and materials. 2-Aryl-1,3-dithiolanes, 2-aryl-1,3-dithiolane *S*-oxides, and 2-aryl-1,3-dithiolane *S,S'*-dioxides were synthesized as previously described.[2] Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, NADPH, and dilaurylphosphatidyl choline were purchased from Sigma Chemical Co. All other reagents and buffers were obtained in the highest quality from commercially available sources. Aminobenzotriazole was a gift of Professor Paul Ortiz de Montellano of this department.

Chiral aryl-1,3-dithiolane *S*-oxides were synthesized by the method of Pitchen *et al.* (22). The optical purity of the purified (*cis*)- and (*trans*)-*S*-oxide products were determined by NMR analysis using a chiral shift reagent.[1] The absorption spectra of pure (*cis*)- and (*trans*)-*S*-oxide were determined in order to relate the optical purity of the products to the absolute configuration correlation of dialkyl *S*-oxides independently correlated by other means (23). Previous studies of thioketal *S*-oxides have established the relationship between the CD sign and the absolute stereochemistry of the dialkyl *S*-oxides (24). A positive CD associated with the absorption between 285 and 195 nm can be correlated with an (*R*)-*S*-oxide configuration (23). Separation of each enantiomer of each diastereomer was accomplished by HPLC with a Chiralcel OD column, as described below. 2-Aryl-1,3-dithiolane *S*-oxides from enzymatic incubations were compared with synthetic chiral *S*-oxides by HPLC for determination of absolute stereochemistry.

Rabbit lungs were obtained from Pel-Freez Biological and were from pregnant (25–30 days of gestation) New Zealand white rabbits. Rabbit lung cytochrome P-450_{IIB-4} was isolated and purified from pregnant female rabbits as previously described (25). Rabbit lung cytochrome P-450_{IIB-4} had a specific content of 14.2 nmol of P-450/mg of protein. Rabbit cytochrome P-450_{IIB-4} had characteristically high benzphetamine *N*-demethylase activity (26) (30.4 nmol/min/nmol of enzyme) and was a single band (M_r 49,500) estimated to be greater than 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (27). Rat cytochrome b_5 and NADPH-cytochrome P-450 reductase were purified to apparent homogeneity by a method previously described (28). The specific content of cytochrome b_5 was 32.8 nmol of b_5/mg of protein and the reductase preparation had an activity of 66 μmol of cytochrome *c*/min/mg of protein in 0.3 M potassium buffer (pH 7.7) at 25°.

Rabbit lung flavin-containing monooxygenase was isolated and purified from pregnant female rabbits by a method previously described (29). Rabbit lung flavin-containing monooxygenase had a specific content of 13.4 nmol of flavin/mg of protein. Rabbit lung flavin-containing monooxygenase had characteristically high *N,N*-dimethylaniline *N*-oxygenase activity (30) (33.6 nmol/min/nmol of protein) and was judged to be homogeneous (greater than 98% pure) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (27).

Metabolic incubations and enzyme assays. *S*-Oxygenation of 2-aryl-1,3-dithiolanes by microsomes, highly purified flavin-containing monooxygenase from rabbit lung, or highly purified cytochrome P-450_{IIB-4} from rabbit lung was measured by monitoring *S*-oxide product formation by HPLC (31). For studies with rabbit lung microsomes, the incubation media contained 50 mM potassium phosphate (pH 8.0), an NADPH-generating system consisting of 0.5 mM $NADP^+$, 2.0 mM glucose-6-phosphate, and 1 IU of glucose-6-phosphate dehydrogenase, and 0.76 mg of rabbit lung microsomes. The reaction was initiated by the addition of substrate (400 μM) and after 5 min the reaction was stopped by the addition of cold CH_2Cl_2 and analyzed for products by HPLC, as described below. For studies with highly purified rabbit lung flavin-containing monooxygenase, the above protocol was used except a buffer consisting of 50 mM potassium phosphate (pH 9.0) was employed. The major phenobarbital-inducible rabbit lung cytochrome P-450 (P-450_{IIB-4}) was purified from rabbit lung by the method of Williams *et al.* (25). Cytochrome P-450_{IIB-4} (0.1 nmol) was reconstituted in the presence of dilaurylphosphatidylcholine (25 μg) and saturating amounts of rat cytochrome P-450 reductase (600 units) and was allowed to stand at 4° for 10 min. Sodium phosphate buffer (50 mM, pH 7.4), the NADPH-generating system, substrate (400 μM final concentration), and rat liver cytochrome b_5 (0.1 nmol) were added for a total volume of 1.0 ml. Incubations were carried out for 10 min at 33° with constant shaking in air and the reactions were terminated and prepared for HPLC analysis as previously described (31).[1,2]

The reactions were stopped by the addition of 2 volumes of cold CH_2Cl_2. After thorough mixing, the insoluble material was separated by a brief centrifugation. After filtration through a 4-μm nylon filter and evaporation, the extract was taken up in acetonitrile for separation and quantitation by HPLC (Rainin system with UV detector set at 240 nm, with a C-18 precolumn and 5-μm C-18 Altex Ultrasphere ODS reversed phase analytical column). The mobile phase (40% CH_3CN/H_2O, v/v) efficiently separates 2-aryl-1,3-dithiolanes, 2-aryl-1,3-dithiolane *S*-oxides, and 2-aryl-1,3-dithiolane *S,S'*-dioxides.[2] The recovery of metabolites as judged by HPLC was greater than 88% and 96% of this material was the dithiolane, dithiolane *S*-oxide, or dithiolane *S,S'*-dioxide. The HPLC traces are remarkably clean and the chromatograms show essentially only starting material and *S*-oxide. This result suggests that other transformations of the dithiolanes are not taking place. Quantitation was accomplished by comparing the integrated area of the dithiolane, dithiolane *S*-oxide, or dithiolane *S,S'*-dioxide HPLC peak after taking into consideration the extinction coefficient values of each species.[2]

After analysis of the reaction products by HPLC as described above, the remaining reaction products were evaporated to dryness and taken up in isopropanol/hexane (18:82, v/v) for separation and quantitation by chiral HPLC [IBM model 9000 with UV detector set at 240 nm, fitted with a Chiracel OD analytical column (25 cm × 0.15 cm, i.d.)

[2] J. R. Cashman, and L. D. Olsen. Stereoselective *S*-oxygenation of 2-aryl-1,3-dithiolanes by the flavin-containing monooxygenase. Submitted for publication.

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from DAICEL Chemical Ind.] This system efficiently separates the starting material and enantiomers of (*cis*)- and (*trans*)-*S*-oxide diastereomers.[3] Quantitation was accomplished by comparing the integrated area for each enantiomer, taking into consideration the extinction coefficient values of each species, as described previously.[3] The concentration of protein was determined by the method of Bradford (32).

Statistics. Data are expressed as the mean ± standard error. Statistical analysis was performed by the Student *t* test for evaluation of the difference between two means.

Results

***S*-Oxygenation of 2-aryl-1,3-dithiolanes.** The biotransformation of 1–5 was studied *in vitro* with pulmonary microsomes, highly purified flavin-containing monooxygenase, and highly purified cytochrome P-450_{IIB-4} from female rabbits. Preliminary studies demonstrated that pulmonary microsomes supplemented with NADPH catalyze the *S*-oxygenation of 2-aryl-1,3-dithiolanes to their *S*-oxides (Table 1). The formation of dithiolane *S*-oxide was a linear function of microsome protein concentration (0–1.4 mg of protein) and of incubation time for at least 7 min. Rabbit lung microsomes that were treated with aminobenzotriazole, a mechanism-based inactivator of cytochromes P-450 (33), inhibited the *S*-oxygenation of 1 and 2 but actually increased the *S*-oxygenation of 3–5 (Table 1). Although the specificity of aminobenzotriazole towards inhibition of pulmonary cytochromes P-450 is not completely understood (34), aminobenzotriazole does not appear to inhibit hepatic or pulmonary flavin-containing monooxygenase-mediated *S*- or *N*-oxygenation (35–37)[1] and in some cases actually stimulates hetero atom oxygenation (34, 35). It is possible that, in addition to its role as an inactivator of cytochromes P-450, aminobenzotriazole may bind to an effector site and stabilize the flavin-containing monooxygenase. The decreased rate of *S*-oxygenation of 1 and 2 in the presence of aminobenzotriazole suggests that rabbit lung microsomal cytochrome P-450 may contribute to *S*-oxygenation of 1 and 2. In order to investigate this aspect more carefully, the *S*-oxygenation of 1 to 5 was investigated with highly purified rabbit lung cytochrome P-450_{IIB-4}.

As shown in Table 2, cytochrome P-450_{IIB-4} efficiently converts 2-aryl-1,3-dithiolanes to their corresponding *S*-oxides. From the data presented, there is not a strong Hammett-type dependence on the electronic character of the aromatic *para*-substituent (38) of the rate of *S*-oxygenation, although it appears that 2-aryl-1,3-dithiolanes with strongly electron-withdrawing *para*-substituents are in general better substrates than 2-aryl-1,3-dithiolanes with electron-donating *para*-substituents (Table 2). Preliminary studies showed that *S*-oxygenation of 1–5 catalyzed by reconstituted cytochrome P-450_{IIB-4} supplemented with NADPH is a linear function of protein concentration (0–0.2 nmol of protein) and of incubation time for at least 10 min. Formation of 2-aryl-1,3-dithiolane *S*-oxide was strictly dependent on the presence of cytochrome P-450 reductase, dilaurylphosphatidylcholine, NADPH, and cytochrome P-450_{IIB-4} but was only modestly dependent on the presence of cytochrome b_5 (data not shown).

The highly purified flavin-containing monooxygenase from rabbit lung catalyzes the NADPH-dependent *S*-oxygenation of 2-aryl-1,3-dithiolanes to their corresponding *S*-oxide (Table 2). Preliminary studies showed that *S*-oxygenation of 1–5 by the highly purified rabbit lung flavin-containing monooxygenase was a linear function of protein concentration (0–0.6 nmol of protein) and of incubation time for at least 5 min. As shown in Table 2, *S*-oxygenation of 1–5 was observed to occur at a uniformly high rate and no apparent Hammett-type dependence (38) on the electronegativity of the dithiolane aryl *para*-substituent was observed. These results are in agreement with other studies that demonstrate that nonaromatic five-membered ring systems that contain a hetero atom are among the best substrates known for the pulmonary rabbit flavin-containing monooxygenase (15, 19, 37).[1] The lack of a Hammett-type substituent dependence (38) of the rate of *S*-oxygenation is similar to that observed for *S*-oxygenation of 2-aryl-1,3-dithiolanes with other flavin-containing monooxygenase enzyme systems, as well as other studies with dialkylsulfides, which suggest that all good substrates for the flavin-containing monooxygenase (8, 39, 40) have similar V_{max} values.

That concurrent nonenzymatic *S*-oxygenation of aryl-1,3-dithiolanes does not contribute to the formation of the *S*-oxides stems from the observations that (a) the ratio of diastereomers remains constant over the time course of the reaction, (b) chemical oxidation (i.e., oxidation of 1–5 to *S*-oxides in the

TABLE 1

S-Oxygenation of 2-aryl-1,3-dithiolanes by rabbit lung microsomes

Substrate		Microsomes + ABT[a]			Microsomes − ABT[a]		
		S-Oxide formed		DE[c]	*S*-Oxide formed		DE
		(cis)-*S*-Oxide	(trans)-*S*-Oxide		(cis)-*S*-Oxide	(trans)-*S*-Oxide	
		nmol/min/mg of protein		%	nmol/min/mg of protein		%
1	4-OCH_3	0.2[d]	2.7 ± 0.2	86	1.0 ± 0.2[e]	3.3 ± 1.2	53
2	4-H	0.1	1.6 ± 0.2	88	2.0 ± 0.3[e]	3.9 ± 1.1[f]	34
3	4-Cl	ND[g]	6.5 ± 0.5	100	0.9 ± 0.1[e]	1.6 ± 0.1[e]	28
4	4-CN	ND	1.3 ± 0.2	100	ND	0.5 ± 0.1[e]	100
5	4-NO_2	0.1	6.1 ± 1.0	96	0.8 ± 0.2[e]	4.3 ± 0.9	69

[a] Rabbit lung microsomes (0.76 mg of protein/incubation) were incubated in the presence of an NADPH-generating system, aminobenzotriazole (ABT), and substrate (400 μM) in potassium phosphate buffer (pH 8.0) at 33° for 3 min. Each value is the average of four determinations ± standard error.

[b] Incubation performed as described above in the absence of aminobenzotriazole.

[c] Product diastereoselective excess [% (trans)-*S*-oxide- % (cis)-*S*-oxide].

[d] The range of values is 0–0.3 nmol/min/mg of protein

[e] $p < 0.001$ versus microsomes plus aminobenzotriazole.

[f] $p < 0.05$ versus microsomes plus aminobenzotriazole.

[g] ND, Not detectable.

[3] J. R. Cashman, L. D. Olsen, and L. M. Bornheim. Enantioselective *S*-oxygenation by flavin-containing and cytochrome P-450 monooxygenases. Submitted for publication

TABLE 2

S-Oxygenation of 2-aryl-1,3-dithiolanes by rabbit lung flavin-containing monooxygenase and cytochrome P-450_{IIB-4}

Substrate		Flavin-containing monooxygenase[a]			Cytochrome P-450_{IIB-4}[b]		
		S-Oxide formed		DE[c]	*S*-Oxide formed		DE
		(cis)-*S*-Oxide	(trans)-*S*-Oxide		(cis)-*S*-Oxide	(trans)-*S*-Oxide	
		nmol/min/nmol of protein		%	nmol/min/nmol of protein		%
1	4-OCH_3	ND[d]	22.3 ± 2.0	100	ND	6.9 ± 1.4	100
2	4-H	ND	24.4 ± 6.1	100	ND	1.0 ± 0.1	100
3	4-Cl	ND	17.1 ± 1.9	100	ND	2.7 ± 0.8	100
4	4-CN	ND	26.4 ± 1.1	100	ND	19.3 ± 0.9	100
5	4-NO_2	NA[e]	NA		ND	9.5 ± 1.8	100

[a] Incubations were performed as described in Experimental Procedures and the values are the average of 4 determinations ± SE

[b] Diastereoselective excess as described in Table 1.

[c] ND, not detectable

[d] NA, not available.

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presence of H_2O_2 or $NaIO_4$) is extremely slow (40) and requires a large concentration of oxidizing agents, and (c) incubations performed in the presence of catalase gave the same stereoselectivity of *S*-oxidation as those incubations performed in the absence of catalase (data not shown). That **1–5** are not oxidized by lipid peroxides or endogenous H_2O_2 during the short incubation times employed can be seen from a comparison of the diastereoselectivity of H_2O_2- or $NaIO_4$ catalyzed *S*-oxygenation of **1–5** and the diastereoselectivity of the microsome-catalyzed *S*-oxygenation of **1–5**. The average percentage diastereoselectivity for $NaIO_4$ and H_2O_2-catalyzed *S*-oxidation of **1–5** is 39 and 31%, respectively (data not shown). For pulmonary microsomes, the average percentage diastereoselectivity for *S*-oxygenation of **1–5** in the presence and absence of aminobenzotriazole is 94 and 57%, respectively (Table 1). The data suggest that nonenzymatic processes do not contribute to *S*-oxygenation of **1–5** and that in the presence of aminobenzotriazole the diastereoselectivity is markedly increased.

***S*-Oxygenation of 2-aryl-1,3-dithiolane *S*-oxides.** The *S*-oxygenation of 2-aryl-1,3-dithiolane *S*-oxides with various rabbit lung monooxygenase preparations was performed in order to determine the products of the reactions as well as to determine the enzymes involved in the transformations. In the presence of rabbit lung microsomes supplemented with NADPH, 2-aryl-1,3-dithiolane *S*-oxides are *S*-oxygenated to the corresponding *S,S'*-dioxide (Table 3). In contrast to initial *S*-oxygenation of dithiolanes, the rate of rabbit lung microsome catalyzed *S,S'*-dioxygenation is significantly decreased and a tendency for a modest Hammett-type substituent dependence (38) of the rate of *S'*-oxygenation is observed. Thus, compounds **6a** and **7a** are *S*-oxygenated at an elevated rate, compared with compounds **9a** and **10a**. A modest Hammett-type substituent dependence of the *S'*-oxygenation of 2-aryl-1,3-dithiolane *S*-oxides is observed for the purified flavin-containing monooxygenase from rabbit lung (Table 3). As observed for rabbit lung microsomes, the rate of *S*-oxygenation catalyzed by purified rabbit flavin-containing monooxygenase of compounds **6a–10a** is significantly decreased, in comparison with the parent sulfide. Compounds **6a**–**8a** are *S*-oxygenated more efficiently than **9a** and **10a**. Incubations performed with **6a–10a** in the presence of rabbit lung cytochrome P-450_{IIB-4} gave variable and only very small amounts of *S,S'*-dioxide products. As a result, the amount of *S,S'*-dioxygenation catalyzed by rabbit cytochrome P-450_{IIB-4} was not quantitated.

TABLE 3

S-Oxygenation of 2-aryl-1,3-dithiolane S-oxides by rabbit lung microsomes and rabbit lung flavin-containing monooxygenase

Each value is the average of four determinations ± standard error.

Substrate		*S,S'*-Dioxide formed			
		Rabbit lung microsomes[a]		Rabbit lung flavin-containing monooxygenase[a]	
		(cis)-*S*-Oxide	(trans)-*S*-Oxide	(cis)-*S*-Oxide	(trans)-*S*-Oxide
		nmol/min/mg of protein		nmol/min/mg of protein	
6a	4-OCH_3	0.9 ± 0.2	2.8 ± 0.4	0.2[b]	2.0 ± 0.3
7a	4-H	ND[c]	4.1 ± 0.6	0.9 ± 0.2	3.6 ± 0.9
8a	4-Cl	0.7 ± 0.2	2.2 ± 0.3	0.9 ± 0.2	1.6 ± 0.3
9a	4-CN	ND	ND	ND	0.1[b]
10a	4-NO_2	0.1[b]	1.9 ± 0.3	0.6 ± 0.1	1.3 ± 0.2

[a] Incubations performed as described in Experimental Procedures.
[b] The range of values is 0–0.2 nmol/min/mg of protein or nmol/min/nmol of protein
[c] ND, not detectable.

In a control experiment, addition of aryl-1,3-dithiolane *S*-oxides **6a–10a** to inactive rabbit pulmonary microsomes or to active microsomes in the absence of NADPH did not result in any transformation of **6a–10a**. This result suggests that reduction or other transformation of **6a–10a** does not occur during the short time period of the incubation. That no aryl-1,3-dithiolane (from reduction) or *para*-substituted benzaldehyde (from oxidation) is observed in the metabolic reactions of **6a–10a** shows that *S*-oxygenation of **6a–10a** is the only detectable biotransformation taking place.

Stereoselectivity of 2-aryl-1,3-dithiolane *S*-oxygenation. As shown in Table 4, a slight stereochemical preference for addition of an oxygen atom by rabbit lung microsomes is to the *pro-S*-sulfur atom for **1** and **2** and to the *pro-R*-sulfur atom for **3** and **4**. Thus, for **2**, addition of an oxygen to the *pro-S*-sulfur atom was found to be 59% (i.e., 40% 1*S*,2*S*; 19% 1*S*,2*R*), with 41% addition of oxygen to the *pro-R*-sulfur atom (i.e., 26% 1*R*,2*R*; 15% 1*R*,2*S*). In the presence of aminobenzotriazole, the increased enantioselectivity for *S*-oxygenation of **2** shows a preference for addition of oxygen to the *pro-S*-sulfur atom (i.e., 64% 1*S*,2*S*), with 36% addition of oxygen to the *pro-R*-sulfur atom (i.e., 36% 1*R*,2*R*). For **3** and **4**, a marked preference for addition of oxygen to the *pro-R*-sulfur atom was observed (i.e., average value of 73% 1*R*,2*R*), with 27% addition of oxygen to the *pro-S*-sulfur atom (i.e., 12% 1*S*,2*S* and 15% 1*S*,2*R*). In the presence of aminobenzotriazole, the enantioselective *S*-oxygenation of **1**, **3**, and **4** was significantly increased (i.e., average value of 88% 1*R*,2*R* and 12% 1*S*,2*S*).

For rabbit lung cytochrome P-450_{IIB-4}, a marked preference for addition of oxygen to the *pro-S*-sulfur atom of **1–4** was observed (i.e., average percentage enantioselectivity of 91% 1*S*,2*S* and 9% 1*R*,2*S*). In strong contrast, almost exclusive preference for addition of oxygen to the *pro-R*-sulfur atom was observed for the highly purified rabbit pulmonary flavin-containing monooxygenase (Table 4). The results clearly demonstrate that rabbit lung cytochrome P-450_{IIB-4} and flavin-containing monooxygenase *S*-oxygenate **1–4** with opposite enantioselectivity.

That nonenzymatic *S*-oxygenation is not occurring can be seen from a comparison of enzymatic versus H_2O_2-catalyzed *S*-oxidation of **1–5**. As shown in Table 4, enantioselective *S*-oxidation of **1–5** is quite large. In contrast, H_2O_2-catalyzed *S*-oxygenation of **1–5** has zero enantioselectivity (data not shown).

Discussion

The overall enzymatic *S*-oxygenation of 2-aryl-1,3-dithiolanes with microsomes or highly purified flavin-containing monooxygenase or cytochrome P-450_{IIB-4} from rabbit lung can be described by the scheme in Fig. 1. Each step has been investigated with five *para*-substituted 2-aryl-1,3-dithiolanes and represents a process that converts the dithiolane to the corresponding dithiane *S*-oxide and, in a second reaction, converts the *S*-oxide to the dithiolane *S,S'*-dioxide. At extremely long reaction times, the *S,S'*-dioxide is not indefinitely stable and is converted to the corresponding benzaldehyde (in a process that presumably involves formation of the dithiolane *S,S,S'*-trioxide) (40), but during the short incubation times employed in this study decomposition of *S*-oxide reaction prod-

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TABLE 4

Stereoselective S-oxygenation of 2-aryl-1,3-dithiolanes by various rabbit lung enzyme preparations

Absolute stereochemistry was determined from reactions shown in Tables 1 and 2, as described in Experimental Procedures. Each value is the average of four determinations ± standard error.

	Substrate	S-Oxide formed[a]											
		Flavin-containing monooxygenase				Cytochrome P-450$_{IIB-4}$				Rabbit lung microsomes[a]			
		(cis)-S-Oxide		(trans)-S-Oxide		(cis)-S-Oxide		(trans)-S-Oxide		(cis)-S-Oxide		(trans)-S-Oxide	
		(1R,2S)[b]	(1S,2R)	(1S,2S)	(1R,2R)	(1R,2S)	(1S,2R)	(1S,2S)	(1R,2R)	(1R,2S)	(1S,2R)	(1S,2S)	(1R,2R)
								%					
1	4-OCH$_3$	ND[c]	ND	0	100	ND	ND	90 ± 3	10 ± 3	75.5 ± 11	24.5 ± 11	56.1 ± 1	43.9 ± 1
2	4-H	ND	ND	3.2 ± 2.0	96.7 ± 2.0	ND	ND	92 ± 2	8 ± 2	43.2 ± 3	56.8 ± 4	60.5 ± 2	39.5 ± 2
3	4-Cl	ND	ND	2.4 ± 1.7	97.6 ± 1.6	ND	ND	90 ± 4	10 ± 4	57.6 ± 8	42.4 ± 9	32.0 ± 2	68.0 ± 2
4	4-CN	ND	ND	1.5 ± 0.3	98.5 ± 0.4	ND	ND	91 ± 3	9 ± 3	ND	ND	26.8 ± 1	73.2 ± 1

[a] Experiment performed in the absence of aminobenzotriazole.
[b] The first designation is for the absolute stereochemistry of the sulfur atom.
[c] ND, Not detectable.

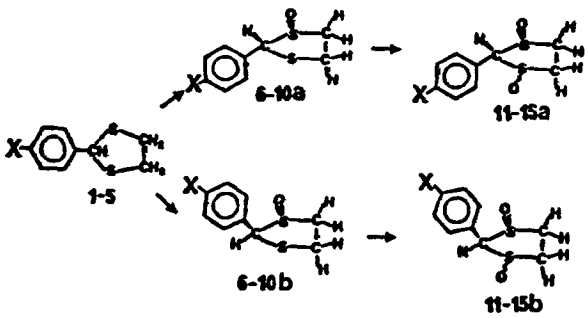

Fig. 1. Overall S-oxidative biotransformation of the 2-aryl-1,3-dithiolanes. The trans-diastereomers and the cis-diastereomers are designated as a and b, respectively.

ucts did not pose a problem and no *para*-substituted benzaldehyde was ever observed.

For the *S*-oxygenation of 2-aryl-1,3-dithiolanes by rabbit lung microsomes, a significant contribution to the *S*-oxygenation of 1 and 2 may be due to cytochromes P-450 whereas 3, 4, and 5 are largely *S*-oxygenated by the flavin-containing monooxygenase. This conclusion is based on the results of the effects of aminobenzotriazole (Table 1) and from the determination of the absolute stereochemistry of the *S*-oxide products obtained (Table 4) (see below). It is possible, however, that another unidentified aminobenzotriazole-sensitive reaction is also in part responsible for *S*-oxygenation of 1 and 2. Although aminobenzotriazole is known to stimulate rabbit lung microsomal oxygenation of substrates of the flavin-containing monooxygenase (34), this does not preclude other processes from contributing to substrate oxidation.

Aminobenzotriazole, a mechanism-based inactivator of cytochromes P-450 (33), significantly inhibits the formation of 6 and 7 from 1 and 2 (Table 1). Stereochemical analysis (Table 1) of the products formed from rabbit lung microsomes demonstrates that, in the presence of aminobenzotriazole, the diastereoselectivity for (*trans*)-*S*-oxide formation increases. For 2, in the absence of aminobenzotriazole, the increased enantioselectivity [i.e., mainly (*pro-S*)-*S*-oxide] is consistent with the involvement of cytochrome P-450 in the *S*-oxygenation (data not shown). That the *S*-oxide enantiomer that is increased in the presence of aminobenzotriazole is mainly the (*pro-R*)-*S*-oxide for 3 and 4 suggests that the flavin-containing monooxygenase is primarily responsible for the *S*-oxygenation of 3 and 4 in rabbit lung microsomes. Compound 1, at least in part, appears to be *S*-oxygenated by cytochromes P-450 but in the presence of aminobenzotriazole 1 is almost exclusively *S*-oxygenated by the flavin-containing monooxygenase [i.e., 97% (*trans*)-(1*R*,2*R*)-*S*-oxide is formed; data not shown]. This may suggest that rabbit lung microsomes contain a cytochrome P-450 that is particularly sensitive to the inhibitory properties of aminobenzotriazole (34) and this isozyme is the major contributor to cytochrome P-450-dependent *S*-oxygenation of 1. Whereas a specific aminobenzotriazole-sensitive cytochrome P-450 isozyme may participate in the *S*-oxygenation of 1, the flavin-containing monooxygenase is largely responsible for *S*-oxygenation of 1 in rabbit lung microsomes.

For both highly purified rabbit lung flavin-containing monooxygenase and cytochrome P-450$_{IIB-4}$, 2-aryl-1,3-dithiolanes 1–5 are efficiently *S*-oxygenated (Table 2). *S*-Oxygenation of 1–5 by the purified monooxygenases is highly diastereoselective for formation of the (*trans*)-*S*-oxide diastereomer. Varying the electronic nature of the *para*-substituent of 2-aryl-1,3-dithiolanes does not influence the rate of flavin-containing monooxygenase-catalyzed *S*-oxygenation. These results are in agreement with other studies that suggest that at least for this class of substrate the rate-determining step occurs after product formation (15). In contrast, cytochrome P-450$_{IIB-4}$ tends to *S*-oxygenate electron-deficient *para*-substituted 2-aryl-1,3-dithiolanes more efficiently than electron-rich *para*-substituted 2-aryl-1,3-dithiolanes in disagreement with other studies employing hepatic enzyme systems (41) (Table 2). The results with cytochrome P-450 tend to support the notion that electron-deficient dialkylsulfides are *S*-oxygenated more efficiently than electron-rich dialkylsulfides (41).

The *S*-oxygenation of 2-aryl-1,3-dithiolane *S*-oxides was investigated with microsomes and highly purified flavin-containing monooxygenase and cytochrome P-450$_{IIB-4}$ from rabbit lung. As shown in Table 3, microsomes and purified flavin-containing monooxygenase are competent to *S*-oxygenate 6a–10a. Attempts to demonstrate cytochrome P-450$_{IIB-4}$-catalyzed *S*-oxygenation of 6a–10a were unsuccessful. From the limited data, it appears that *S*-oxide *S'*-oxygenation is largely dependent on flavin-containing monooxygenase activity, although compared with *S*-monooxygenation the rate of *S,S'*-dioxide formation is markedly decreased. In contrast to *S*-monooxygenation, *S,S'*-dioxygenation is somewhat sensitive to the nature of the *para*-

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substituent, demonstrating a Hammett correlation (i.e., ρ value of 0.3 and 0.27 for microsomes and purified flavin-containing monooxygenase from rabbit lung, respectively) (Table 3). Based on the results presented, a distinction between a single-electron transfer mechanism and an anionic nucleophilic mechanism for *S*-oxygenation cannot be made solely on the basis of Hammett-type correlations (40, 43). Stereochemical analysis of the products of *S,S'*-dioxygenation, in principle, could provide insight into enzyme mechanism. However, the relative instability of the *S,S'*-dioxides precluded careful examination of the absolute stereochemistry of **11**–**15**. Absolute stereochemistry assignments of *S*-monooxide metabolites of **1**–**4** were investigated and this has led to an understanding of rabbit pulmonary monooxygenase mechanism.

A summary of the results of *S*-oxygenation catalyzed by microsomes and purified cytochrome P-450_{IIB-4} and flavin-containing monooxygenase from rabbit lung is shown in Tables 1, 2, and 4. From the results shown in Tables 1 and 2 it is apparent that the *S*-oxygenation of **1**–**5** is highly stereoselective and the (*trans*)-*S*-oxide product is formed in large excess of the (*cis*)-*S*-oxide. For rabbit lung microsomes the diastereoselective excess was modest and varied between 100% and 28%, with an average value of 57% (Table 1). In the presence of aminobenzotriazole, a mechanism-based inactivator of cytochromes P-450 (33) and an agent that does not inhibit pulmonary flavin-containing monooxygenase activity (34, 37), a marked increase in diastereoselectivity is observed. The diastereoselective excess varied between 100% and 86%, with an average value of 94%. We interpret these results to suggest that the contribution to dithiolane *S*-oxygenation from rabbit lung cytochrome P-450 is significant and the low diastereoselective excess observed for lung microsomes in the absence of aminobenzotriazole reflects an involvement of cytochrome P-450 in (*cis*)-*S*-oxide formation. For the 2-aryl-1,3-dithiolanes studied, both the purified flavin-containing monooxygenase and the purified cytochrome P-450_{IIB-4} from rabbit lung catalyze exclusive formation of the (*trans*)-*S*-oxide (Table 2). Because the flavin-containing monooxygenase catalyzes only (*trans*)-*S*-oxide formation of **1**–**5** this suggests that some other monooxygenase [presumably cytochrome P-450 IVA4 (pg-ω) or IVB1 (form 5), which are the other major isoenzymes present] catalyzes formation of the (*cis*)-*S*-oxide of **1**–**5**.

As shown in Table 4, the absolute stereochemistry of *S*-oxygenation of **1**–**4** catalyzed by microsomes, flavin-containing monooxygenase, and cytochrome P-450_{IIB-4} from rabbit lung was determined. It is clear that rabbit lung flavin-containing monooxygenase has a marked preference for forming (*trans*)-(*1R,2R*)-*S*-oxides. In contrast, cytochrome P-450_{IIB-4} *S*-oxygenates **1**–**4** to produce exclusively (*trans*)-(*1S,2S*)-*S*-oxides. From inspection of the stereochemistry of *S*-oxide products from experiments employing rabbit lung microsomes, it is apparent that cytochrome P-450 is preferentially responsible for *S*-oxygenation of **2**, whereas flavin-containing monooxygenase activity is mainly responsible for *S*-oxygenation of **1**, **3**, **4**, and **5**. Thus, attack by the peroxyflavin of rabbit lung flavin-containing monooxygenase is directed to the *pro-R*-sulfur atom, whereas attack by the cytochrome P-450 iron-oxo species is directed to the (*pro-S*)-sulfur atom. It is possible that the 2-aryl-1,3-dithiolanes are *S*-oxygenated by rabbit pulmonary cytochrome P-450 in an "edge-on" orientation with respect to the dithiolane group and the heme moiety, whereas rabbit pulmonary flavin-containing monooxygenase requires alignment of the dithiolane substrate in a deep narrow cleft where steric interactions between the peroxyflavin and the aryl group constrain the substrate into a *trans*-orientation (Fig. 2). Compared with hog liver flavin-containing monooxygenase, which can produce (*cis*)-2-aryl-1,3-dithiolane *S*-oxide products as well as (*trans*)-2-aryl-1,3-dithiolane *S*-oxide products (40),[1] it would appear that the rabbit lung flavin-containing monooxygenase is highly stereoselective and presumably possesses a much smaller binding pocket than the hepatic form of the enzyme. That a large degree of enantioselectivity is observed for both monooxygenase-catalyzed reactions suggests that, if single-electron transfer reactions are on the reaction pathway, collapse of active oxygen species with sulfide cation radical is more rapid than rearrangement or isomerization of a sulfur cation radical, at least for this class of substrate (43). However, an alternative mechanism involving nucleophilic attack via anionic mechanisms is favored for the flavin-containing monooxygenase (40, 42).

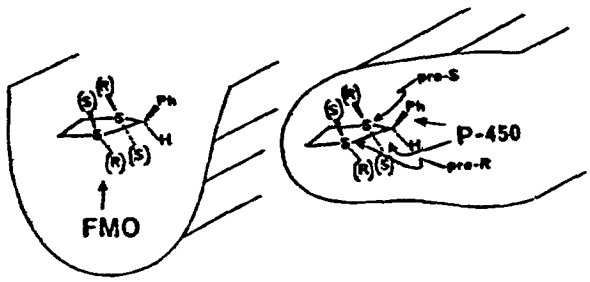

Fig. 2. *S*-oxygenation of 2-aryl-1,3-dithiolanes by cytochrome P-450-catalyzed "edge-on" *S*-oxygenation and flavin-containing monooxygenase (*FMO*)-catalyzed *S*-oxygenation.

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References

1. Damani, L. A. Thioethers, thiols, dithioic acids and disulfides: Phase I reactions, in *Sulfur-Containing Drugs and Related Organic Compounds* (L. A. Damani, ed.), Vol. 1, Part A. Ellis Horwood, Chichester, 135–150 (1989).
2. Massel, P., J. F. Henderson, and J. Axelrod. *S*-Demethylation by microsomal enzymes. *J. Pharmacol. Exp. Ther.* 143:1–6 (1964).
3. Mozier, N. M., K. P. McConnel, and J. L. Hoffman. *S*-Adenosyl-L-methionine: thiomethyl *S*-Methyltransferase, a new enzyme in sulfur and selenium metabolism. *J. Biol. Chem.* 263:4527–4531 (1988).
4. Hanzlik, R. P. Prediction of metabolic pathways: sulfur functional groups, in *Foreign Compound Metabolism* (J. Caldwell and G. D. Paulson, eds.). Taylor and Francis, London, 65–78 (1984).
5. Duggan, D. E. Sulindac: therapeutic implications of the prodrug/pharmacophore equilibrium. *Drug Metab. Rev.* 12:325–337 (1981).
6. Renwick, A. G. Sulfoxides and sulfones, in *Sulfur-Containing Drugs and Related Organic Compounds* (L. A. Damani, ed.), Vol. 1, Part B. Ellis Horwood, Chichester, 133–154 (1989).
7. Williams, K. I. H., S. H. Burstein, and D. S. Layne. Metabolism of dimethylsulfide, dimethylsulfoxide and dimethysulfone. *Arch. Biochem. Biophys.* 117:84–87 (1966).
8. Ziegler, D. M. Functional groups bearing sulfur, in *Metabolic Basis of Detoxication* (W. B. Jakoby, J. R. Bend, and J. Caldwell, eds.), Academic Press, New York, 171–184 (1982).
9. Cashman, J. R., and S. Peña. Canrenone formation via general-base-catalyzed elimination of 7α-(methylthio)spironolactone *S*-dioxide. *Chem. Res. Toxicol.* 2:109–113 (1989).
10. Hucker, H. B., P. M. Ahmad, and J. K. Miller. Absorption, distribution and metabolism of dimethyl-sulfoxide in the rat, rabbit and guinea pig. *J. Pharmacol. Exp. Ther.* 154:176–184 (1966).
11. Damani, L. A., and A. A. Houdi. Cytochrome P-450 and FAD-monooxygenase mediated *S*- and *N*-oxygenations. *Drug Metab. Drug Interact.* 6:350–363 (1988).
12. Poulsen, L. L. Organic sulfur substrates for the microsomal flavin-containing monooxygenase. *Rev. Biochem. Toxicol.* 3:33–49 (1981).

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13. Egan, R. W., P. H. Gale, W. J. A. Vanden Heuvel, E. M. Baptiste, and F. A. Kuehl. Mechanism of oxygen transfer by prostaglandin hydroperoxidase. *J. Biol. Chem.* 255:323–326 (1980).
14. Hunt, P. A., S. C. Mitchell, and R. H. Waring. Some properties of sulfoxidizing enzymes, in *Biological Reactive Intermediates. II. Chemical Mechanisms and Biological Effects* R. Snyder, D. V. Parke, J. J. Kocsis, D. J. Jollow, G. G. Gibson, and C. M. Whitmer, eds.) Plenum, New York, 1255–1262 (1982).
15. Ziegler, D. M. Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds, in *Enzymatic Basis of Detoxication* (W. B. Jacoby, ed.). Academic Press, New York, 201–227 (1980).
16. Light, D. R., D. J. Waxman, and C. T. Walsh. Studies on the chirality of sulfoxidation catalyzed by bacterial flavoenzyme cyclohexanone monooxygenase and hog liver flavin adenine dinucleotide containing monooxygenase. *Biochemistry* 21:2490–2498 (1982).
17. Waxman, D. J., D. R. Light, and C. T. Walsh. Chiral sulfoxidations catalyzed by rat liver cytochromes P-450. *Biochemistry* 21:2499–2507 (1982).
18. Levi, P. E., and E. Hodgson. Stereospecificity in the oxidation of phorate and phorate sulphoxide by purified FAD-containing monooxygenase and cytochromes P-450 isozymes. *Xenobiotica* 18:29–39 (1988).
19. Williams, D. E., D. M. Ziegler, D. J. Nordin, S. E. Hale, and B. S. S. Masters. Rabbit lung flavin-containing monooxygenase is immunologically and catalytically distinct from the liver enzyme. *Biochem. Biophys. Res. Commun.* 125:116–122 (1984).
20. Tynes, R. E., P. S. Sabourin, and E. Hodgson. Identification of distinct hepatic and pulmonary forms of microsomal flavin-containing monooxygenase in the mouse and rabbit. *Biochem. Biophys. Res. Commun.* 126:1068–1075 (1985).
21. Ohmiya, Y., and H. M. Mehendale. *N*-Oxidation of *N,N*-dimethylaniline in the rabbit and rat lung. *Biochem. Pharmacol.* 32:1281–1285 (1983).
22. Pitchen, P., E. Dunach, M. N. Deshmukh, and H. B. Kagan. An efficient asymmetric oxidation of sulfides to sulfoxides. *J. Am. Chem. Soc.* 106:8188–8193 (1984).
23. Mislow, K., M. M. Green, P. Laur, J. T. Melillo, T. Simmons, and A. L. Ternay, Jr. Absolute configuration and optical rotary power of sulfoxides and sulfinate esters. *J. Am. Chem. Soc.* 87:1958–1976 (1965).
24. Auret, B. J., D. R. Boyd, E. S. Cassidy, R. Hamilton, F. Torley, and A. F. Drake. Structure and absolute stereochemistry of thioacetal *S*-oxides obtained by fungal metabolism of 2-aryl-1,3-dithiolanes. *J. Chem. Soc. Perkin Trans. I* 2827–2829 (1985).
25. Williams, D. E., S. E. Hale, R. T. Okita, and B. S. S. Masters. Prostaglandin-ω hydroxylase cytochrome P-450 purified from pregnant rabbit lung. *J. Biol. Chem.* 259:14600–14608 (1984).
26. Mazel, P. *Fundamentals of Drug Metabolism and Drug Disposition* (B. N. LaDu, H. G. Mandel, and E. L. Way, eds), Williams and Wilkins, Baltimore, 546–550 (1972).
27. Laemmi, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T$_4$. *Nature (Lond.)* 227:680–685 (1970).
28. Waxman, D. J., and C. T. Walsh. Phenobarbital-induced rat liver cytochrome P-450 *J. Biol. Chem.* 257:10446–10457 (1982).
29. Williams, D. E., S. E. Hale, A. S. Muerhoff, and B. S. S. Masters. Rabbit lung flavin-containing monooxygenase: purification and induction during pregnancy *Mol. Pharmacol.* 28:381–390 (1985).
30. Ziegler, D. M., and F. H. Petit. Formation of an intermediate *N*-oxide in the oxidative demethylation of *N,N*-dimethylaniline catalyzed by liver microsomes. *Biochem. Biophys. Res Commun* 15:188–193 (1964).
31. Cashman, J. R., and J. Proudfoot. A reverse phase high pressure liquid chromatographic assay for flavin-containing monooxygenase activity. *Anal. Biochem* 175:274–280 (1989).
32. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254 (1976)
33. Ortiz de Montellano, P. R., and J. M. Mathews. Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. *Biochem. J.* 195:761–764 (1981).
34. Mathews, J. M., and J. R. Bend. *N*-Alkylaminobenzotriazoles as isozyme-selective suicide inhibitors of rabbit pulmonary microsomal cytochrome P-450. *Mol. Pharmacol.* 30:25–32 (1986).
35. Cashman, J. R., and D. M. Ziegler. Contribution of *N*-oxygenation to the metabolism of MPTP by various liver preparations. *Mol. Pharmacol.* 29:163–167 (1986).
36. Cashman, J. R., and S. Peña. *S*-oxygenation of 7α-thiomethylspironolactone by the flavin-containing monooxygenase. *Drug Metab. Drug Interact.* 6:337–348 (1988).
37. Williams, D. E., M. Shigenaga, and N. Castagnoli, Jr. The role of cytochromes P-450 and flavin-containing monooxygenase in the metabolism of (*S*)-nicotine by rabbit lung. *Toxicologist* 9:24 (1989).
38. Lowry, R. H., and K. S. Richardson. *Mechanism and Theory in Organic Chemistry*, 2nd Ed. Harper and Row, New York, 130–142 (1981).
39. Ziegler, D. M. Metabolic oxygenation of organic nitrogen and sulfur compounds, in *Drug Metabolism and Drug Toxicity* (J. R. Mitchell and M. G. Horning, eds), Raven Press, New York, 33–53 (1984).
40. Cashman, J. R., J. Proudfoot, Y.-K. Ho, M. S. Chin, and L. D. Olsen. Chemical and enzymatic oxidation of 2-aryl-1,3-oxathiolanes: mechanism of the hepatic flavin-containing monooxygenase. *J. Am. Chem. Soc.* 111:4844–4852 (1989).
41. Watanabe, Y., T. Iyanagi, and S. Oae. Kinetic study on enzymatic *S*-oxygenation promoted by a reconstituted system with purified cytochrome P-450. *Tetrahedron Lett.* 21:3685–3688 (1980).
42. Ziegler, D. M. Flavin-containing monooxygenases: catalytic mechanism and substrate specificities. *Drug Metab. Rev* 19:1–32 (1988).
43. Pryor, W. A., and W. H. Hendrickson. Reaction of nucleophiles with electron acceptors by S_N2 or electron transfer (ET) mechanisms: *tert*-butyl peroxybenzoate/dimethyl sulfide and benzoyl peroxide/*N,N*-dimethylaniline systems. *J. Am. Chem. Soc.* 105:7114–7122 (1983).

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